Anatomical specificity of the action of testosterone in relation to the regulation of birdsong and the underlying neuroplasticity

By

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Abstract

One important goal of the field of behavioral neuroscience is to develop theories about the relative importance of different parts of the central and peripheral nervous systems in the modulation of behavior. Using canaries, a well-studied songbird, this thesis investigates the different levels at which testosterone acts to regulate song behavior and the underlying neuroplasticity. Given the integral role hormones play in the modulation of birdsong, the distinct sites at which androgen receptors are distributed throughout the songbird brain and periphery, and the distinct roles specific nuclei play in regulating different features of song, songbirds like canaries are especially amenable to such an investigation. The results of this thesis demonstrate testosterone's role in the regulation of birdsong and neuroplasticity is pleiotropic: the regulation of specific song features, such as the motivation to sing versus song quality, and the neuroplasticity of the circuitry that regulates birdsong, is dependent on where and how (genomic versus non-genomic-like) in the songbird brain and periphery testosterone acts. For instance, testosterone action at the syrinx, the avian vocal organ, plays a different role in the regulation of birdsong compared to its central substrate, the song control system. Within the song control system, the action of testosterone within the sensorimotor region HVC (acronym is name) plays a different regulatory role of song than the motor region called the robust nucleus of the arcopallium. Many of these song features were regulated by the rapid actions of estrogens, a key product of the aromatization of testosterone in the regulation of birdsong. This suggests that estrogens are key in the regulation of song over very short time scales. Lastly, testosterone drove changes in plasticity in the song control system in a direct (acting within specific sites) as well as in an indirect (by

enhancing singing activity) manner. This pleiotropic regulation by testosterone also applies to highly socio-sexually relevant vocal signals including in the songs called trills. These results highlight the complex role played by steroid hormones in the coordination of various suites of behaviors into a functional, adaptive response.

I went to the woods because I wished to live deliberately, to front only the essential facts of life, and see if I could not learn what it had to teach, and not, when I came to die, discover that I had not lived. I did not wish to live what was not life, living is so dear; nor did I wish to practice resignation, unless it was quite necessary. I wanted to live deep and suck out all the marrow of life, to live so sturdily and Spartan-like as to put to rout all that was not life, to cut a broad swath and shave close, to drive life into a corner, and reduce it to its lowest terms.

Henry David Thoreau, *Walden: Or, Life in the Woods (1854)*

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Chapter 1: Introduction

1.1 Birdsong: Brief Introduction

Birdsong is species-typical, stereotypic set of usually long, learned, complex vocalizations produced mostly by males (Ball and Hulse, 1998; Ball et al 2003;Brainard and Doupe, 2013; Catchpole and Slater 2008; Konishi,1985). The term birdsong is reserved for a type of vocalization produced in the context of mate choice and territory defense by species in the suborder Passeres (i.e. the oscines) of the order Passeriformes (Catchpole and Slater 2008; Konishi, 1985). All species in this order have at least one member of each sex that produce song and based on data collected to date they all appear to possess a specialized neural substrate for learning and producing these elaborate vocalizations (Brainard and Doupe, 2013; Jarvis et al., 2005; Konishi, 1985; Pfenning et al., 2014). The issue of female song is somewhat complex. For many years it was assumed that males only sing in most species (Odom et al. 2014). However more recent studies have revealed that female song is quite widespread and is observed especially in tropical species where males and females will duet as well as in many species in Australasia (Odom et al. 2014). The causes and functions of female song are not as well understood as compared to male song (Odom et al., 2014) and we will focus on male song in this dissertation.

One of the most intriguing aspects of singing in songbirds from the temperate zone is its seasonal regulation. For instance, when photoperiods are long in the spring and early summer, males experience a robust increase in song as opposed to when photoperiods are short in the fall and winter, when they tend

to sing much less (Ball, 1999; Ball et al., 2002; Dawson et al., 2001; Farner and Wingfield, 1980; Hurley et al., 2008). Along with increases and decreases in day length are concomitant changes in the concentrations of circulating testosterone (T), which increases the probability that song will be produced (Dawson et al., 2001; Hurley et al., 2008; Smith et al., 1997). Although decades of work have been dedicated to studying the neural and hormonal regulation of birdsong, it is unclear exactly where in the brain and periphery T acts to regulate song and what aspects of song behavior are regulated by T.

In many songbird species song exhibits a high degree of systematic intraspecific geographic variation that has been labeled as a phenomenon akin to language dialects in humans (Jenkins, 1978; Marler and Tamura, 1962, 1964). This intraspecific variation was one of the first clues that strongly suggested that birdsong might be learned (Ball and Hulse, 1998). Song is indeed learned in a manner akin to human language (Brainard and Doupe, 2000, 2013; Konishi, 1985; Konishi, 2004; Lipkind et al., 2013; Marler, 1977; Marler, 1991; Marler, 1997; Nottebohm, 1984; Tchernichovski and Marcus, 2014; Waser and Marler, 1977). Song learning and production are regulated by gonadal steroids that can act on the discrete network of interconnected brain nuclei, known as the song control system, that control the activity of the avian vocal production organ, the syrinx (Ball et al., 2002; Brainard and Doupe, 2013). These characteristics make songbirds an excellent model taxon in which to study the anatomical, hormonal, and neural regulation of a complex learned motor behavior (Ball et al., 2002; Brainard and Doupe, 2013; Fee and Scharff, 2010). In this thesis proposal, I will

first review some of what we know about the regulation of brain and behavior in songbirds in relation to vocal production, with special emphasis on the hormonal regulation of song. Then I will describe the multiple experiments I have completed to investigate the multiple ways by which T can regulate song production in songbirds. Overall, these experiments illuminate our understanding of the complex ways in which T acts throughout the songbird brain and periphery to coordinate the different features of song into an adaptive output.

1.2 Anatomy of the song control system

Songbirds possess a neural specialization that has evolved in concert with their complex vocal behaviors. This specialization is called the song control system and consists of a group of interconnected brain nuclei that regulate the learning, production, and perception of song (Brainard and Doupe, 2013; Brenowitz, Perkel, and Osterhout, 2010; Nottebohm, Stokes, and Leonard, 1976; Nottebohm, 1982). The song system was first discovered in canaries (*Serinus canaria*) by Nottebohm (Nottebohm et al., 1976). The song system has been characterized based on a wealth of studies in many songbird species, but primarily conducted in species such as zebra finches (*Taeniopygia guttata*) as well as canaries, that live well in the laboratory (see Zeigler and Marler 2008 for reviews). The song control system can be divided into a pathway involved in motor control located in the caudal telencephalon and a pathway involved in vocal learning and plasticity located in the anterior forebrain (Figure 1).

In the caudal telencephalon, the motor pathway begins with a projection from HVC (acronym is proper name) to the robust nucleus of the arcopallium

(RA). While it was originally thought that the functions of HVC were distributed across the nucleus in a unitary fashion (Margoliash et al., 1994), more recent work has shown that the function of HVC in the regulation of song is highly topographical. For instance, the medial portion of HVC coordinates the syntactical rules of song while the lateral portion of HVC regulates the lexical components of song. The middle portion of HVC seems to play less of a role overall in regulating these features but may partially be involved in regulating syntactical rules (Basista et al., 2014). Notably, there seems to very little to no possibility of exchange of information from these discrete portions of HVC as they project to RA, suggesting highly independent functions of HVC (Basista et al., 2014), similarly to how in humans distinct yet partially overlapping portions of the cortex encode syntactical and lexical components of speech independently (Menenti, Segaert, and Hagoort, 2012).

RA is also quite topographical. RA projects to the dorsomedial portion of the intercollicularis (DM-ICo) and both RA and DM-ICo project to the tracheosyringeal part of the nucleus of the twelfth cranial nerve (nXIIts) which projects to the syrinx. Vicario (1991) has shown that projections from RA to the nXIIts are myotopically organized: projections are sent from RA to distinct portions of nXIIts that in turn also control distinct muscles in the syrinx. In addition to regulating the muscles of the syrinx via nXIIts, RA also projects to respiratory nuclei in the hindbrain such as nucleus retroambigualis (Ram) and nucleus parambigualis (Pam), which coordinate respiratory activity with song

production (Ashmore, Renk, and Schmidt, 2008; Schmidt, McLean, and Goller, 2012).

Located in the anterior forebrain is the circuit critical for song learning and auditory feedback on song and behavioral plasticity (Brainard and Doupe, 2000; Fee and Goldberg, 2011). In the anterior forebrain, this pathway begins with a projection from HVC to Area X of the medial striatum, which projects to the medial part of the dorsolateral anterior thalamic nucleus (DLM). DLM then projects to the lateral magnocellular nucleus of the anterior nidopallium (LMAN), which in turn projects to RA and back to Area X. The projections from Area X to DLM to LMAN and back to Area X make a striatal-thalamic-cortical loop, similarly to what has been observed in mammalian brains (Fee and Goldberg, 2011). The projection from LMAN to RA is thought to be critical for adaptive variability exploration involved in learning song as well as maintaining song in adulthood if auditory feedback is impaired (Doupe and Kuhl, 1999; Kao, Doupe, and Brainard, 2005; Kojima, Kao, and Doupe, 2013; Stepanek and Doupe, 2010; Woolley, Rajan, Joshua, and Doupe, 2014). Importantly, projections from the HVC all the way down to the syrinx are unilateral and the left and right syringeal halves function independently from one another (Brainard and Doupe, 2013; Konishi, 1985; Schmidt et al., 2012). However, information regarding song is still exchanged between the hemispheres, via projections from RA to Pam, where Pam sends projections to the thalamic nucleus Uvaeformis (Uva) bilaterally, and Uva sends projections to HVC unilaterally (Margoliash, 1997a; Schmidt et al., 2012). Indeed, results from Akutagawa and Konishi (2005) suggest that Uva

functions as a hub in coordinating neural input into the SCS. These projections illustrate the importance of integrating multiple modalities for the normal production of song.

While early studies clearly showed auditory feedback was critical for song learning, it was not until the mid-nineties that brain regions responsible for this began to be elucidated (Brainard and Doupe, 2013). The general organization of the auditory system in avian species is similar to what has been described in mammals: auditory information enters through the cochlea, is transduced by thalamic nuclei (nucleus ovoidalis in birds), then to the midbrain (nucleus mesencephalicus lateralis pars dorsalis in birds), and is then further processed by cortical nuclei. These cortical nuclei begin with Field L, which is organized in layers similar to the primary auditory cortex in mammals (Margoliash, 1997a; Mooney, 2009). Information from Field L proceeds to nuclei in the secondary auditory cortex, including the caudomedial nucleus of the nidopallium (NCM) and the caudomedial nucleus of the mesopallium (CMM). NCM and CMM are bidirectionally connected. CMM, via multiple pathways (Figure 1) projects to HVC (Remage-Healey, 2012). The projections from NCM and CMM that eventually converge onto HVC are thought to underlie the template formed during song learning and critical for encoding neural information that is responsible for neurons that fire selectively to the bird's own song (BOS) (Remage-Healey, 2012). The template or song memory is probably formed in NCM and then conveyed to HVC (Bolhuis and Moorman, 2015). HVC's position in the SCS makes it a viable candidate of being able to provide that song memory into the

song system (Bolhuis and Moorman, 2015; Bolhuis, Okanoya, and Scharff, 2010).

1.2.1 The medial preoptic area and the regulation of song

Recent evidence suggests that an area outside of the classically defined SCS is involved in regulating whether or not songbirds will sing. The first piece of evidence to suggest that this might be the case was shown in the original study describing the song system by Nottebohm et al (1976). In this study, following bilateral lesions to the HVC of a male canary, the male could not produce song, but he still *tried* to sing and engaged in all of the associated motor movements but could not produce a clear species-specific song (Nottebohm et al 1976). After Nottebohm and colleagues administered exogenous T to this bird it increased its singing-related behaviors vigorously, but still could not produce audible song. Although not widely recognized at the time, this finding suggested that there is a separation between brain areas where steroid hormones act to initiate song as compared to where they might modulate the quality of song produced. One candidate brain region for regulating the motivation to sing is the medial preoptic nucleus (POM), a well-defined area within the preoptic area. The preoptic area has been known to be important in the regulation of male-typical sexual behaviors since the pioneering work of Larsson (Larsson and Heimer, 1964; Heimer and Larsson, 1967). In birds such as Japanese quail, studies by Balthazart, Panzica, and Ball have clearly implicated the POM in the regulation of all behavior related to male-typical sexual behavior (Ball and Balthazart, 2010; Panzica, Viglietti-Panzica, and Balthazart, 1996).

Studies conducted by Riters and colleagues have demonstrated that the POM is required for the production of sexually-motivated song in songbirds. For example, after bilateral electrolytic lesions to the POM of male European starlings (*Sturnus vulgaris*) these songbirds exhibited a drastic decrease in song output (Riters and Ball 1999). Notably these effects were specifically affected by the POM, as lesions outside of this area left this courtship behavior intact. Algers and Riters (2006), using the same lesion techniques described above, showed that POM lesions reduced song output and nest-box directed behaviors in starlings during a sexual context and Alger colleagues (2009) showed via the same lesions that this disrupts immediate-early gene labeling in brain nuclei of the social behavior network, including the periaqueductal gray (PAG) and ventral tegmental area (VTA). These data suggest that the POM regulates the motivation to sing differently depending on the sexual context and its function is tied to activity in other brain regions involved in affective state.

1.3 Seasonal and hormonal regulation of song and neuroplasticity

The periodic change in behavior, including reproductive behaviors, of non-human animals associated with the seasons of the year has been appreciated at least since Aristotle (Foster and Kreitzman, 2009). Rowan is credited as the first to formally test the idea that changes in photoperiod governed these changes in vertebrate animals based on his studies of wild Juncos (Rowan, 1925). This was followed by a series of studies such as those by Bissonnette (1930) demonstrating the rapid stimulation of gonad growth caused by long days in

European starlings. Now with the myriad studies on photoperiodism and behavior it is clear that day length exerts many of its effects through modulation of the hypothalamo-pituitary-gonadal (HPG) axis, which it turn stimulates reproductive physiology and behavior (Ball and Balthazart, 2014; Ball et al., 2002; Barker, et al., 2013; Dawson et al., 2001; Hurley et al., 2008). The regulation of song behavior and neuroplasticity in songbirds is one of the best examples of this phenomenon (Ball et al., 2002; Farner and Wingfield, 1980; Nottebohm, 1981; Wingfield, Jacobs, and Hillgarth, 1997; Tramontin and Brenowitz, 2000).

Song is a reproductive behavior and thus it is not surprising that its occurrence correlates positively with the onset of the breeding season in many species (Ball, 1999; Ball et al., 2002; Catchpole, 2003; Catchpole and Slater 2008; Dawson et al., 2001; Farner and Wingfield, 1980; Hurley et al., 2008; Wingfield et al., 1997). In temperate-zone songbirds, photoperiod length has a significant effect on birds' breeding state. For instance, when days are short during autumn songbirds exhibit a regressed HPG; however, when hours of daylight increase in length during spring the HPG-axis undergoes recrudescence (Dawson et al 2001). This recrudescence leads to an increase in plasma gonadal steroid hormone concentrations that is thought to underlie the increase in the output of courtship behaviors that occurs during this time. Numerous studies have shown a causal role for T in regulating song output (Ball et al 2003; Schlinger and Brenowitz 2002). For example, one early study demonstrated that castrated male zebra finches undergo a reduction in song output and the associated courtship behaviors, both of which can be restored upon treatment

with exogenous T administration (Pröve, 1974). Similar findings have been shown in a variety of songbird species (Arnold, 1975; Harding et al., 1988; Walters andCollado, 1991; Sartor, Balthazart, and Ball, 2005).

Until recently, no studies had formally tested where in the songbird brain seasonally increasing T acts to regulate song; uncritical assumptions were made, however, claiming that T acts in the songbird brain to stimulate singing behavior without any mention of the specific brain regions or of the song features under regulation (Sakata and Vehrencamp, 2012). Arnold (1981), however, alluded to the fact that, given the distinct functions of each nucleus within the SCS, it is possible that hormones like T act in a non-redundant manner to regulate song. Few attempts have been made in the ensuing 30 years to investigate this question, perhaps because of the complexity required for studies that try to isolate separate effects of T in a variety of brain structures. However, some data relevant to this general question of non-redundant action has been collected. For example, Meitzen and colleagues (2007) have shown that T acts within HVC to regulate at least one feature of song, acoustic stereotypy. For instance, white-crowned sparrows (*Zonotrichia leucophrys)* treated with an AR antagonist in HVC showed no reduction in song rate but sang songs that had lower acoustic consistency through successive song renditions. This study is in line with observations made by Brenowitz and Lent (2002), who showed castrated white-crowned sparrows treated with T solely in their HVC did not sing. These results are not surprising considering the results showing that canaries treated with large amounts of T with lesioned HVC still attempt to sing and show all of the normal

postures associated with song but cannot produce audible song (Konishi, 1985; Nottebohm et al., 1976). Thus, T may act outside of the SCS proper to modulate the motivation to produce song.

Numerous investigations indicate that it is not T *per se* that regulates songbird courtship behavior at the cellular level, but its metabolites such as 5α-dihydrotestosterone (5α-DHT) and 17β-Estradiol (E2) acting through their cognate receptors, AR and ER, respectively (Ball et al., 2002; Ball et al 2003; Harding, 2004; Wingfield et al., 1997). These receptors when bound by their ligand will act as transcription factors to change gene expression and lead to the suite of biochemical changes underlying the observed behavioral and morphological changes caused by these hormones. Studies analyzing the effects of the metabolites of T on birdsong have mostly employed zebra finches. In a study by Harding et al (1983) castrated male zebra finches treated with various combinations of T, androstenedione, E2, and 5α-DHT restored song output to normal levels, in contrast to birds treated with non-aromatizable androgens, which were ineffective for stimulating song output. Subsequently confirming the effects of aromatizable androgens, Walters et al (1988) treated zebra finches with the aromatizable androgen, androstenedione with or without the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD) and found treatment with ATD abolished the song-restoring effects of androstenedione.

Seasonal changes in the song control system

Concordant with the robust seasonal changes in song behavior observed in songbirds are equally robust morphological and physiological changes in the

SCS nuclei. As with changes in song, seasonal changes in the SCS can be driven by T (Reviewed in Ball and Balthazart and Balthazart 2002, Ball et al., 2002, Ball et al., 2004), but can also occur independently of T in response to other endogenous and exogenous cues (see below). Changes in a number of attributes in the SCS are the result of these T-induced effects. For instance, Nottebohm (1981) showed that the volumes of the key song nuclei, Area X, HVC, and RA are almost double their size in the spring compared to the fall. Other attributes that change seasonally and may contribute to these volumetric changes are: cell size, density, and number (Brenowitz et al., 1991; Johnson and Bottjer, 1993); and dendritic and synaptic morphology (DeVoogd et al., 1985; DeVoogd and Nottebohm, 1981; Doupe, 1994). Alterations in one or more of these attributes are hypothesized to underlie the seasonal and T-induced changes in Area X, HVC, and RA. For HVC, it has been confirmed that these enhancements in volume, at least in part, are due to the incorporation of new neurons arising from radial glial cells residing in the ventricular zone (Alvarez-Buylla, Ling, and Nottebohm, 1992; Goldman et al., 1996), among other changes such as angiogenesis (Louissant et al., 2002), in addition to changes in cell spacing and cell size (Bottjer and Johnson, 1995).

The obvious candidate for regulating these SCS nuclei changes is T. Indeed, castrated songbirds show a decrease in the SCS nuclei volumes reminiscent of the regression in size observed during the fall (Brown and Bottjer, 1993; Nottebohm, 1981; Sartor et al., 2005; Smith et al., 1997). Moreover, T plays a critical role in multiple phases of new neuron proliferation, recruitment,

and incorporation. For instance, Barker et al. (2014) showed that T enhances the proliferation of cells in the ventricular zone of the telencephalon in adult canaries. These results are in line with what has been observed by Williams and colleagues (1999), who showed that estrogens promote the initial migration of new neurons from the ventricular zone (Williams et al., 1999). Yamamura et al. (2011) showed that T and its androgenic and estrogenic metabolites have a synergistic relationship in enhancing the recruitment as well as the differentiation of new neurons into HVC as measured by the immunoreactivity of the microtubulue associated protein, doublecortin, a marker of new neurons (Balthazart et al., 2008; Balthazart and Ball, 2014). It is hypothesized that T exerts many of its effects on the incorporation and survival of new neurons in HVC via brain-derived neurotrophic factor (BDNF) (Louissant et al, 2002; Reviewed in Chen et al., 2013).

More recent studies have begun to pinpoint where T is acting to modulate these neural changes. Brenowitz and Lent (2002) showed that implanting T unilaterally in the HVC of castrated white-crowned sparrows caused not only an increase in the volume of the HVC ipsilateral to the implant but also an increase in the volume of the ipsilateral RA, suggesting T can transsynaptically affect neuroplasticity. Therefore, T acting directly within the SCS can have important effects on neuroplasticity throughout this circuit.

While seasonal plasticity in the SCS is caused directly by T, T-independent factors can also modulate plasticity in this circuitry (Ball et al., 2002). For instance, Bernard et al. (1997) reported that photoperiod, even in

castrated American tree sparrows, can modulate plasticity in the SCS. Also, Larson and colleagues (2013) have shown that neural activity in RA can retrogradely affect neural plasticity in HVC, such that GABA receptor stimulation in RA causes a decrease in the number of new neurons incorporated into HVC. Moreover, the act of singing also affects the SCS, as in canaries singing activity has been shown to correlate with the volume of HVC (Alvarez-Borda and Nottebohm, 2002; Hurley et al., 2008) as well as the expression of brain-derived neurotrophic factor (BDNF) and the incorporation of new neurons into this nucleus (Li et al., 2000). The social context also can affect neuroplasticity in the SCS, in that being in a competitive situation and not being able to sing is associated with a smaller HVC volume in starlings (Sartor and Ball, 2005) and a lack of social enrichment has been shown to reduce the volume of HVC as well the number of new neurons incorporated into the HVC in zebra finches (Adar et al., 2008). Importantly, while all of these factors can act independently, the effects can be modulated by the presence of T, as has been shown in a study by Alvarez-Borda and Nottebohm (2002) in which the presence of gonads and singing activity played separate yet additive roles when regulating neuroplasticity of the SCS. It should be noted, however, that it has been difficult to ascertain the effects of gonad-independent factors in regulating SCS neuroplasticity because the presence of T acting globally has been a confound (Adkins-Regan, 2005).

1.4 Distribution of AR, ER, and aromatase in the song control system

Following discoveries about the neural substrates controlling song, in light of the observations made regarding the hormonal regulation of song,

investigations on possible sites where these hormones may act to regulate song commenced. One of the first studies was performed by Arnold and colleagues (1976), in which castrated male zebra finches were injected with tritiated T following by autoradiographic localization of what cells exhibit uptake of the isotope. The results of these studies showed that T accumulated in the same basic areas of the non-songbird and mammalian counterparts, such as the septal, hypothalamic, and midbrain regions. However, T also accumulated in multiple SCS nuclei, such as HVC, MAN, RA, DM-ICo, and nXIIts. Later, it was shown that androgens also have high-affinity binding sites in the syrinx (Lieberburg and Nottebohm, 1979). However, because tritiated T could be converted to DHT or E2 it was difficult to determine which brain regions expressed androgen receptors or estrogen receptors or both. Hence, later studies using different techniques such as in situ hybridization and immunohistochemistry were conducted and largely in line with these early results and additionally showed respiratory nuclei such as Ram expressed androgen receptors (AR) (Balthazart, et al., 1992; Bernard et al., 1999; Gahr, 1990; Metzdorf, Gahr, and Fusani, 1999). ER of the alpha subtype (ERα) is expressed in hypothalamic and limbic structures as well as in the ICo of non-songbirds and songbirds; additional ERα binding sites are also present in song nuclei such as HVC in some species (Balthazart, Gahr, and Surlemont, 1989; Bernard et al., 1999; Gahr, Flügge, and Güttinger, 1987; Gahr, Guttinger, and Kroodsma, 1993). ER of the beta subtype (ERβ) is located in the ICo and POM (Bernard et al., 1999).

As mentioned above the POM seems to be involved in the motivation to sing. This area shows dense expression of AR and ERα,β (Balthazart et al 1989, 1992; Ball et al 1999; Foidart et al 1999; Bernard et al 1995). Moreover, while the POM expresses such a high density of aromatase immunoreactivity that this immunoreactivity defines the boundaries of the POM in a manner consistent with standard histochemical definitions (Balthazart *et al* 1996; Riters *et al* 2000), there is very little or no expression of this enzyme in the song control nuclei themselves (Ball et al 2003). However, aromatase expression is found densely throughout the auditory regions such as NCM and CMM, indicating these regions may produce estrogens that act on the SCS (Balthazart, et al., 1996; Saldanha et al., 2000; Shen, et al., 1995). This hypothesis has garnered support based on recent work showing estrogens produced in the NCM rapidly transform BOS selectivity in HVC of anesthetized zebra finches (Remage-Healey and Joshi, 2012). These data also provide support for the idea that estrogens produced in the brain may modulate behavior on a faster time-scale, in a manner akin to neuromodulation or a neurotransmitter's mechanism (i.e., non-genomic, acting via second messenger cascades or ion channel modification) (Cornil et al., 2013; Cornil, Ball, and Balthazart, 2012; Remage-Healey, 2012)

2.1 The medial preoptic nucleus and sexual motivation

As has been suggested above, T acting in an area or areas outside of the traditionally-defined SCS are probably involved in regulating the motivation to sing. One candidate site for this action is the POM, as Riters et al (1999) showed

that POM lesions greatly reduce song output in male starlings and disrupt neural activity in regions like the VTA which are involved in affective states (Alger, Maasch, and Riters, 2009). However, only studies in non-songbirds have been conducted testing the effects of T in the POM on male sexual motivation.

For instance, many studies indicate that T action in the POM is critical in regulating sexual motivation in males, including sexual motivation in birds such as Japanese quail (*Coturnix japonica*; Ball and Balthazart 2010). The function of the POM and T action in this region in regulating sexual motivation in quail has been well characterized. The POM of quail is larger in males than in females as long as males are exposed to higher T and this larger volume is associated with the expression of male-typical sexual behaviors (Panzica et al 1991, 1996; Thompson and Adkins-Regan, 1994). Androgen and estrogen receptor mRNA and protein are expressed densely in this region (Balthazart et al 1989, 1992; Ball et al 1999; Foidart et al 1999) in quail and the POM is also rich in aromatase (Balthazart et al 1990; Foidart et al 1995). Peripheral blockade of aromatase in castrated male quail treated with T abolishes sexual motivation (Balthazart et al 1997) and POM lesions cause a significant reduction in the time males spend near or looking at a female, measure of sexual motivation (Balthazart et al 1998). These data suggested that T could exert its effects on stimulating sexual motivation via its conversion to estrogen by aromatization in the POM. This hypothesis was supported, as castrated male quail treated with T located in the POM showed normal measures of sexual arousal (Riters et al 1998) and this effect is absent if the birds were treated with an aromatase inhibitor (Watson and

Adkins-Regan 1989). It is therefore probable that T is working in a similar way in songbirds to regulate the motivation to sing in males.

2.2 Indirect Connections between the POM and the song control system

For T action in the POM to stimulate the sexually motivated song, one would assume that it has to somehow modulate the song system, since activity in this circuit is required for song production. There is no evidence to date that indicates the POM has direct connections to the song control system; Riters and Alger (2004) found that POM neurons synapse at an area near RA, but this is as close to direct connections as has been described to date. However, the POM projects to areas of the brain that do connect to the song control system, such as the ventral tegmental area (VTA), periaqueductal gray (PAG), locus coreleus (LoC), and DM-ICo (Appeltants et al 2000; Riters and Alger 2004). Evidence that supports these indirect connections as influencing song output, is the fact that lesions of the POM in starlings that reduce sexually motivated song also reduce immediate-early gene labeling in the VTA and PAG (Algers et al., 2009).

If these connections are involved in regulating sexually-motivated song, this would be in line with previous investigations in rodents and quail indicating that connections between the POM and midbrain nuclei are involved in regulating sexual behavior. For instance, Brackett and Edwards (1984) and Edwards and Einhorn (1986) demonstrated in rats that connections between the midbrain tegmentum and POM are essential for sexual motivation. Specifically, by using a unilateral preoptic lesion combined with a contralateral lesion of the medial forebrain bundle (MFB) rats showed a reduction in sexual behavior, including

sexual motivation. Axons from the POM pass through the MFB and synapse onto the central gray and dorsolateral and ventral portions of the tegmentum. As indicated by studies in rodents (Pfaff 1968; Sheridan 1978) and quail (Carere et al 2007), efferents from the POM to the MFB are a possible route through which steroid-hormone sensitive cells in the POM could influence sexual motivation.

3. The current thesis: Investigating the multiple levels at which testosterone regulates birdsong and underlying neuroplasticity

Throughout the history of psychology and neuroendocrinology, researchers have postulated the importance of different parts of the central and peripheral nervous systems in the modulation of behavior. For instance, the pioneering physiological psychologist Frank Beach (1971) hypothesized that androgens acting in the periphery, such as at the mammalian penis, as opposed to actions in the brain, played a more important role in regulating sexual arousal. Others have also adopted a similar view (Sachs, 2007). While most researchers adopt the view that hormonal action in the brain is critical for the regulation of these behaviors, some of the points indicated by researchers like Beach and Sachs are well taken, such as that peripheral actions may feed back to modulate behavior. Indeed, researchers like Pfaff have proposed that the best way to attack questions of the neuroendocrine regulation of behavior is to use a reverse engineering approach, partitioning the regions involved in the distinct behavioral components into isolated avenues of scientific inquiry. For instance, the influence of estrogens acting in the spinal cord and flanks have been fount to be just as important as estrogenic actions in the ventromedial nucleus of the hypothalamus

(VMN), and the results can be combined to provide a comprehensive view of the estrogenic regulation of lordosis in rodents. Indeed, Pfaff and colleagues (2008) have applied this method to the lordosis circuit in female rodents and had great success, allowing one to address how estrogens regulate attractivity versus proceptivity versus receptivity in female rats.

By employing a methodological approach very similar to that employed by Pfaff (2008), the goal of this thesis is to investigate the levels at which T is acting in the songbird brain to regulate song in songbirds, specifically in male canaries. Given the integral role hormones play in the modulation of birdsong, the distinct sites at which androgen receptors are distributed throughout the songbird brain including the SCS, and the distinct roles each of these nuclei play in regulating different features of song, songbirds like canaries are especially amenable to such an approach. Moreover, songbirds, especially canaries, have been extremely well-studied in terms of their behavior, life history, and neuroanatomy, making them a highly tractable study species for this thesis (Ball et al., 2002; Brainard and Doupe, 2013; Dietzen, et al., 2006; Hinde and Matthews, 1957; Hinde and Warren, 1959; Leitner et al., 2001; Nottebohm, 1981, 1984, 1996; Vallet, Beme, and Kreutzer, 1998; Voigt et al., 2003).

Overall, this thesis includes a collection of studies using a variety of experimental techniques, such as social context manipulations, hormone implantation, hormone receptor blockade, and enzyme inhibition. The first experiment will investigate how social cues contribute to song and new neuron incorporation into HVC, both of which are highly dependent on T. This

experiment is especially relevant as, while the effects of photoperiod in the regulation of T's effects on singing and SCS plasticity have been studied extensively, the effects of social cues on these processes are less clear. The next five experiments will test the roles T and AR in the syrinx, POM, HVC, and RA in the regulation of different song features such as the motivation to sing, acoustic features, song consistency, and the regulation of specific components of songs, called trills. The last experiment investigates how the conversion of T to estradiol via aromatase regulates singing on a short timescale to elucidate how song may be regulated by the non-genomic actions of estradiol with special emphasis on key brain regions where these actions may be important. By in large, the results of these experiments will help to enhance our understanding of how hormones act in different regions of the brain and periphery to coordinate distinct features of a complex, learned behavior into a functional response.

Figure Legend:

Figure 1:

Schematic of the SCS. Acronyms described in main text. Courtesy of Heather Williams.

Figure 2:

Diagram showing the distribution of sex steroid hormone receptors in the songbird brain. AR-Androgen receptor; ER-Estrogen receptor. Figure obtained from Ball et al., (2006)

Figure 3:

Autoradiographic images from *in situ* hybridization depicting the distribution of brain regions expressing aromatase. Figure obtained from Forlano, Schlinger, and Bass (2006)

Figures:

Figure 1:

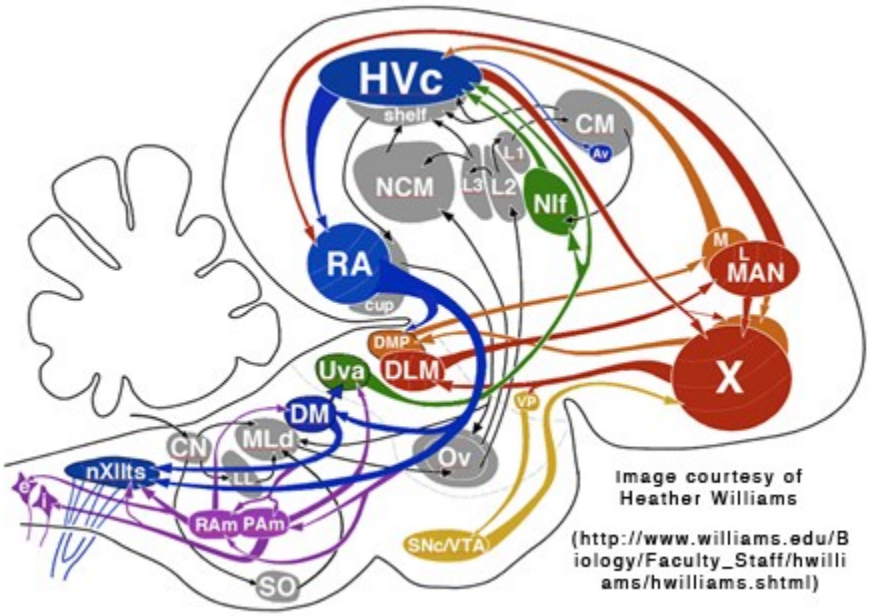


Figure 2:

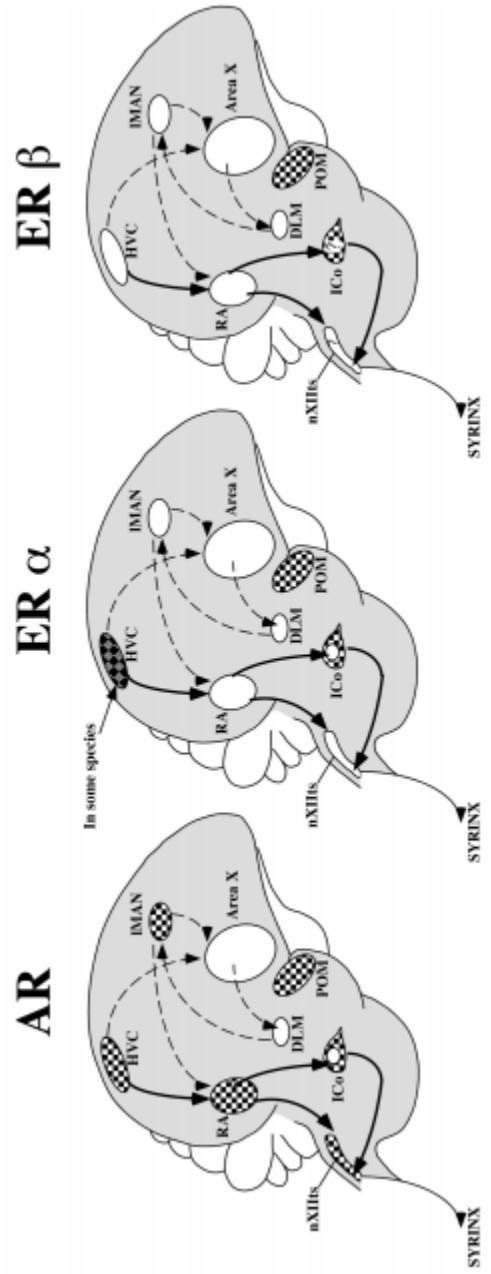
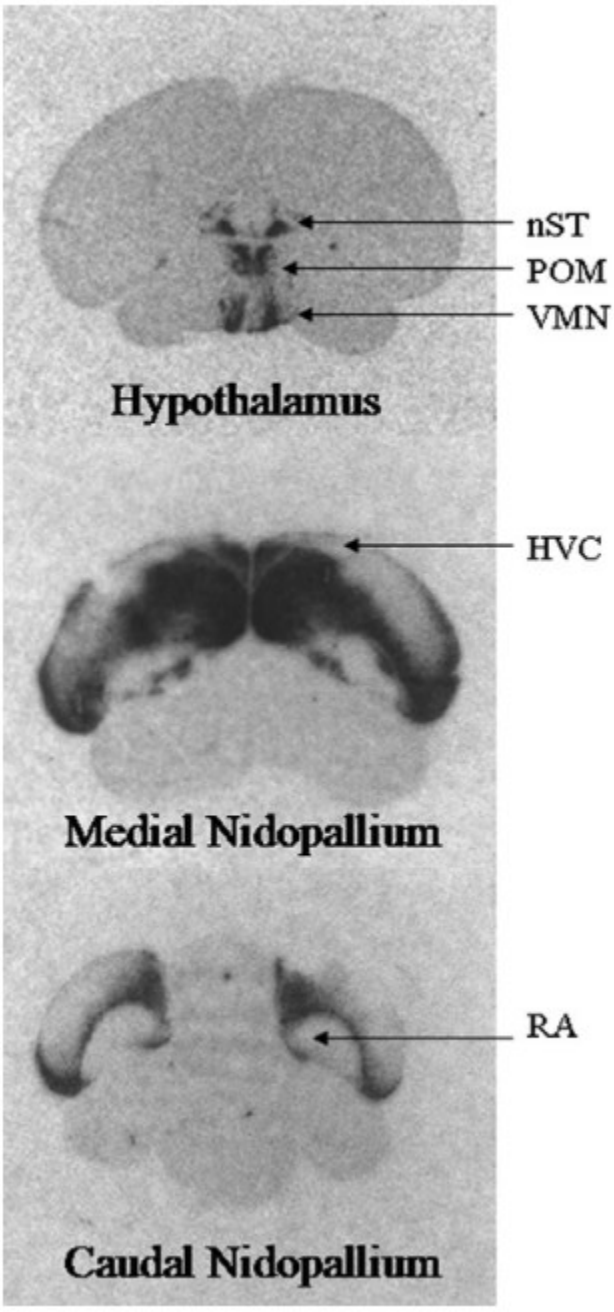

AR
ER α
ER β
HVC
IMAN
Area X
DLM
POM
RA
ICo
nXIIts
SYRINX
In some species

Figure 3:


nST
POM
VMN
Hypothalamus
HVC
Medial Nidopallium
RA
Caudal Nidopallium

Chapter 2: Dissociable effects of social context on song and doublecortin immunoreactivity in male canaries

Title: Dissociable effects of social context on song and doublecortin immunoreactivity in male canaries

Running Title: Social-induced neuroplasticity

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Abstract

Variation in environmental factors such as day length and social context greatly affects reproductive behavior and the brain areas that regulate these behaviors. One such behavior is song in songbirds, which males use to attract a mate during the breeding season. In these species the absence of a potential mate leads to an increase in the number of songs produced, while the presence of a mate greatly diminishes singing. Interestingly, although long days promote song behavior, producing song itself can promote the incorporation of new neurons in brain regions controlling song output. Social context can also affect such neuroplasticity in these song control nuclei. The goal of the present study was to investigate in canaries (*Serinus canaria*), a songbird species, how photoperiod and social context affect song and the incorporation of new neurons, as measured by the microtubule-associated protein, doublecortin (DCX), in HVC, a key vocal production brain region of the song control system. We show that long days increased HVC size and singing activity. In addition, male canaries paired with a female for two weeks showed enhanced DCX-immunoreactivity in HVC relative to birds housed alone. Strikingly, however, paired males sang fewer songs that exhibited a reduction in acoustic features such as song complexity and energy, compared to birds housed alone, which sang prolifically. These results show that social presence plays a significant role in the regulation of neural and behavioral plasticity in songbirds and can exert these effects in opposition to what might be expected based on activity-induced neurogenesis.

Introduction

The social environment causes robust changes in behavior (Cacioppo et al, 2000; Catchpole and Slater, 2003; Conaway, 1971; Lehrman et al,, 1961). For instance, in many species male songbirds sing more songs when in the absence of a potential mate as compared to when the female is present (Orr and Hansell, 1975; Cuthill, 1985; Sockman *et al.*, 2005; Carere *et al.*, 2006; Alward *et al.*, 2013). This suggests that once a male attracts a mate, it is economically favorable to reduce courtship singing (Sockman et al, 2005).

Songbirds possess an interconnected system of brain nuclei that orchestrate the learning and production of song (Figure 1A; reviewed in Nottebohm, 1996; Bottjer and Johnson, 1997; Brenowitz *et al.*, 1997; Fee and Scharff, 2010). The sensorimotor nucleus HVC projects to the robust nucleus of the arcopallium (RA), which is part of the pathway required for song production. HVC also projects to Area X (in the basal ganglia; Bottjer and Johnson, 1997; Fee and Scharff, 2010; Fee and Goldberg, 2011), which is part of the pathway required for song learning and auditory feedback. These nuclei show remarkable plasticity in response to seasonally changing T (Brenowitz and Lent, 2002; Nottebohm, 1981; Thompson, Bentley, and Brenowitz, 2007; Tramontin and Brenowitz, 2000; Ball, 1999). There is also evidence that singing activity (Alvarez-Borda and Nottebohm, 2002; Ball et al 2006; Larson et al., 2013; Nottebohm, 2002; Sartor and Ball, 2005), social cues (Boseret et al 2006; Tramontin et al 1999b), and photoperiod (Bernard et al 1997; Gulledge and Deviche, 1998) can contribute to this seasonal neuroplasticity in the song control

system (SCS) independently of T. For example, Boseret and colleagues (2006) have shown that males housed with a female possess larger HVC with a larger volume relative to males housed with a male. Males housed with a female also had more cells in HVC labeled with doublecortin (DCX), a largely specific marker of new neurons during their first 30-40 days of post-mitotic life (Jacques Balthazart, Boseret, Konkle, Hurley, and Ball, 2008), as compared to those housed with a male, suggesting changes in the rate of neurogenesis. However, this experiment was not designed to allow one to determine whether the female partner enhanced or the male partner attenuated the incorporation of these new neurons (Balthazart et al., 2008). In canaries, singing *per se* also induces neurogenesis and enhances the size of the SCS nuclei (Alward *et al* 2013; Li et al., 2000) but observed changes in neurogenesis could not be driven by singing activity since in the previous experiments males housed with a female sang much less than males housed with another male (Boseret *et al* 2006).

In the present study, we took advantage of the variation in socially modified singing behavior and neuroplasticity in canaries to examine the relationship between photoperiod, social context, behavioral plasticity, and new neuron incorporation. Specifically, we employed behavioral and immunohistochemical techniques to evaluate the effects of photoperiod and social context on song output, acoustic complexity, and DCX-immunoreactivity (-ir) in HVC of male canaries.

Methods And Materials

Subjects

A total of fifteen male and five female border canaries were purchased from a local supplier in July 2010 when they were naturally photorefractory (based on the fact that they were experiencing the ambient photoperiod). They were brought into the laboratory and group housed on short day lengths (8L:16D; SD) for six weeks in cages (1m by 0.5m by 0.5m) in either male or female groups. This photoperiodic treatment has been shown to induce photosensitivity in photorefractory starlings and canaries (Dawson et al, 2001; Hurley et al., 2008). Food and water were provided *ad libitum* for the duration of the experiment. After two weeks in the lab, all birds were laparotomized under isoflurane (3-4% induction, then 1-2% maintenance) to confirm their sex and evaluate their gonadal state; all birds were observed to have regressed testes/oviduct. At the termination of each experiment, the body cavities were examined to confirm gonadal state. The protocols and procedures were approved by the Johns Hopkins University Animal Care and Use Committee and in accordance with the guidelines of the National Institutes of Health.

Experimental procedure

This experiment was performed with five stimulus females and fifteen males divided into three treatment groups. Two groups consisted of male canaries placed individually on long days (14L:10D; LD) in sound-attenuated testing chambers (0.94m x 0.56m x 0.56m) for two weeks to stimulate

reproductive development (Dawson et al., 2001; Hurley et al., 2008) while the other group remained on SD. In one LD group, each male was paired with a female (Paired, n=5) on the day he was transferred to LD, and in another LD group, males were housed alone (Alone, n=5). The third group consisted of male birds housed alone, but kept on SD (n=5). Auditory recordings were collected during the first 2 hours of the morning period on days 1, 2, 3, 7, and 14 after the transfer of the birds to the sound-attenuated chambers. On the final day, the birds were euthanized with an overdose injection of secobarbital (60 mg/kg, i.m.) followed by rapid decapitation. The length and width of the largest testis was measured following decapitation to determine the breeding conditions of the birds. Testis volume was determined using the equation for an ellipsoid (oblate spheroid): $V=4/3\pi a^2 b$, where a is half the width and b is half the length (Stevenson and Ball, 2010).

Song analysis

Each isolation chamber was fitted with a microphone (BT-MP8087 Mini microphone from BandH) and camera (KPC-600 Pinhole Camera 3.6mm from BandH) that was connected to a computer running DVRserver (V6.33b; Mammoth Technologies, Austin, TX) designed for real-time full-motion video-capture and high-speed recording. Each day, the DVRserver captured the dawn singing behavior produced between the hours of 7:30 AM and 9:30 AM in .wav files sampled at 22,050Hz which translated to a frequency range of 0 kHz to 11 kHz. Song files were run through a highpass filter set to a threshold of 900Hz to remove low frequency noise and converted to a digital format using

Goldwavetm (Version 5.55; GoldWave, St. John's, Newfoundland, Canada) before they were visualized into sound spectrograms using Avisoft (SASlab Pro, Berlin Germany), a Windows application for investigating animal acoustic communication by increasing the efficiency in extensive sound analysis projects. For the spectrograms, the fast Fourier transforms length was set to 512 with an overlap of 75% for the temporal resolution. The song parameters of interest for each bird were exported into Excel after being counted by Avisoft with automated parameter measurements. The parameters selected for song analysis were based on previously published work in canaries (Alward *et al* 2013; Voigt and Leitner, 2008) with slight modifications based on past observations in our laboratory. In brief, songs were defined as having a duration greater than one second of continuous notes with gaps no longer than half a second. Each song was verified by looking at the original sonograms to further eliminate noise that escaped the filter. For each bird the song measures included song rate (total number of songs divided by the total recording time; i.e., 10 hours) and the average length of songs.

In addition to the above measures, we were interested in particular features of song such as how loudly the bird sings and song complexity. Previous work suggests that the social context and breeding conditions can modulate acoustic features of song such as amplitude (Cynx and Gell, 2004). To quantify these features, we used Avisoft Sound Analysis Software to compute the following:

Energy: This is the sum of the squared amplitude of a song multiplied by its sampling time (volt^2*second). It quantifies how loud or intense the song is.

Entropy variance: Entropy is a measure of the spectral width and uniformity of a signal. It is a pure number (i.e. unitless) with 0 being a pure tone (e.g., a uniform sinusoidal wave) and 1 being noise (i.e. a non-uniform random signal) (O Tchernichovski, Nottebohm, Ho, Pesaran, and Mitra, 2000). The variance of this measure collapsed across a single bout of singing is indicative of the diversity of vocalization types included. High entropy variance tends to indicate songs with a high degree of vocal variability caused by the inclusion of transitions between multiple different syllable/phrase types in individual songs.

In the current chapter, and all of the following chapters, we analyze multiple features of the canary song that have been shown to be sensitive to changes in T, relevant to changes in the SCS functioning, or important in social interactions in general (Madison et al., 2014; Meitzen et al., 2007, 2009; Rouse and Ball, 2015; Suthers et al., 2012; Tchernichovsky et al., 2000; Voigt and Leitner 2008) (see Appendix). For instance, we will assess acoustic features of song that include amplitude and acoustic frequency as component variables. We are aware of the observation that amplitude and acoustic frequency have been shown to have positive relationships with one another (Zollinger et al., 2012). We realize that when making inferences about the distinct roles of specific brain

regions in regulating various acoustic features of birdsong that one should consider this covariance. However, in the majority of the experiments presented in this thesis dissociations were revealed between changes in measures of song amplitude and acoustic frequency. Moreover, we measure the amplitude and acoustic frequency from songs with an omnidirectional microphone placed in the cage. One may pose the valid question whether the distance of the bird from the microphone may contribute to changes in amplitude and frequency measures. Based on extensive observations of the birds included in the experiment, the production of song takes place in a highly stereotyped manner, wherein the bird will sing from the middle of the perch assuming the species-typical posture. While it has been observed that some birds sing from spots other than the middle of the perch, this was not due to any systematic variations in treatment. Moreover, most of our changes in amplitude and frequency are rather dramatic, suggesting that even if position of singing influences acoustic readout, it would not be enough to explain the substantial variance we observed in most of our experiments due to treatment. Therefore, while we acknowledge the complexities regarding acoustic analysis, we do not feel the possible confounds that can exist between measures of acoustic characteristics apply to the studies presented in this thesis.

Brain extraction and sectioning

The brains were quickly extracted and fixed in 0.5% acrolein solution (9.5 ml 1M phosphate buffered saline, PBS, and 500 µl acrolein) for 2 hours at room temperature. The brains were washed four times for fifteen minutes each in 1M PBS and then transferred to 30% sucrose solution overnight. Once saturated,

brains were flash frozen in powdered dry ice for 5 minutes, then placed at -80°C until brain sectioning. Brains were sectioned in the coronal plane by a single individual to ensure consistency in orientation of sections with a cryostat at 30 µm thickness into three series and we collected every section.

Doublecortin immunocytochemistry and quantification

We used a DCX immunocytochemistry protocol previously used in canary brains (Yamamura, Barker, Balthazart, and Ball, 2011). Sections were washed in 0.01M PBS three times, once in 0.1% sodium borohydride in 0.01M PBS, and 3 times in 0.01M PBS with 1% Triton X (PBST). Endogenous peroxidases were blocked using 0.6% H_2O_2 in PBST for 20 minutes which was followed by three washes in PBST and additional blocking using 10% normal horse serum (NHS) in PBST for 30 minutes. Sections were incubated at 4°C in 2% NHS and PBST and primary antibody (1:5,000 Horse-anti goat, Doublecortin, Santa Cruz, cat#; sc-8066). Sections were washed three times in PBST, then incubated in avidin biotin horseradish-peroxidase complex (Vectastain ABC Elite Kit, 1:200) for 1 hour, and washed three times in PBST. The peroxidase was then visualized using diaminobenzidine (Sigma Fast DAB) for 5 minutes and sections were subsequently washed in 0.01M PBS and mounted onto gelatin-coated microscope slides. Slides were serially dehydrated in ethanol and placed in xylene for 10 min before being coverslipped using Permount (Fisher, Fair Lawn, NJ).

We counted the two different types of DCX-ir cells (Figure 3A)--round and fusiform--in HVC. The round cells are new neurons that have migrated to their final site and begun differentiating while the fusiform cells are still migrating and have not begun differentiating. The counting method was similar to that used by others (Jacques Balthazart et al., 2008; Yamamura et al., 2011). Specifically, DCX-ir cells were counted at three different rostro-caudal levels in three separate fields positioned in the center of HVC and in the adjacent nidopallium lateral and ventral to HVC (see Figure 1 in Balthazart *et al.,* 2008). These three rostro-caudal levels of HVC were roughly equally spaced in the nucleus to provide an overall representation of DCX-ir cells in this structure (Balthazart *et al.,* 2008*)* and they show significant levels of neuronal incorporation in adulthood (Balthazart et al., 2008; Boseret, Ball, and Balthazart, 2007; Kirn, Fishman, Sasportas, Alvarez-Buylla, and Nottebohm, 1999; Yamamura et al., 2011). Immunoreactive cells were manually counted on images digitized through the microscope (20 X objective) in a standardized square area (200 X 200 μm) in each brain region of interest. The Cell Counter function of ImageJ software (version 1.40g; Wayne Rasband, National Institutes of Health) was used to identify DCX-ir cells, which were then classified as round or fusiform by a human observer. The area used for quantification was positioned within the structure of interest in a standard manner using clearly defined brain landmarks as previously described in detail (Jacques Balthazart et al., 2008; Yamamura et al., 2011). All immunoreactive cells that contained a clear unstained nucleus surrounded by stained cytoplasm were manually labeled. Cells were counted on one side of the brain that was

randomly chosen. We also counted the two cell types in comparable brain regions, lateral and ventral to HVC, to confirm specificity of changes in HVC. Cells counts in each area (HVC, lateral or ventral to this nucleus) were added across the three rostro-caudal level (i.e., in three 200 X 200 µm fields or 3 x 0.04= 0.12 mm^2) and expressed as numbers of cells per mm^2.

Nissl staining and HVC and POM area reconstruction

One series of sections was used for a Nissl stain. To obtain an estimate of HVC size, we traced the borders of the HVC at its largest point using Image J (NIH) and computed the corresponding area. The focus of this paper is to assess the effects of social environment and photoperiod on new neuron incorporation as assessed by DCX immunoreactivity. However, we also thought an estimate of HVC size among the groups would be useful. Due to some damage to the dorsal surface of the Nissl-stained sections, we were not able to reliably assess total HVC volume but we could reliably assess HVC size using an area estimate at its largest extent. We also determined the size of the medial preoptic nucleus (POM) as a proxy for central exposure to T (Alward et al., 2013; Charlier, Ball, and Balthazart, 2008; L V Riters et al., 2000). We traced the borders of the POM at the level of the anterior commissure where it fully intersects with the occipitomesencephalicus (OM) tract and computed its area with the use of the Image J software. We confirmed consistency in the planes of sections of all brains by measuring the volume of the nucleus rotundus, a nucleus that has been shown not to change as a function of endogenous or exogenous changes, at the same rostro-caudal level as where the POM was measured. We confirmed

that its size was not different across all treatment groups (One-way ANOVA, $F(2,14)=0.1$, $p>0.9$).

Statistical analyses

All data including the song parameters mentioned above, the number of DCX-ir round and fusiform cells, and the HVC areas were analyzed using one- or two-way ANOVAs. Tukey's post-hoc analyses were used to make pairwise comparisons when appropriate. Differences were considered significant for $p \leq 0.05$.

Like most studies in non-human animals, we took precautions to ensure our studies included sufficient statistical power. Nonetheless, at rare times in this thesis the sample size in some experimental groups fell to four or and even more rarely to three birds. We are well aware of the issues that go along with extrapolating the results of statistical analyses that use small sample sizes (Nakagawa and Hauber, 2011). However, in all cases where small sample sizes may have presented a problem in extrapolation, the results were in line with the experiments in chapters that preceded it or in line with the results obtained by others. Overall then, while small sample sizes were present in some of the experimental groups in this thesis, we are still confident in the reliability of our results given strong a priori hypotheses.

Results

Effects of treatment on breeding physiology

There was a main effect of treatment on testis volume ($F(2,14)=11.43$; $p=0.002$; Figure 1B). Specifically SD males had smaller testis than Alone ($p=0.001$) and Paired ($p=0.04$) males. Alone and Paired males were not statistically different from one another ($p=0.15$). Moreover, there was a main effect of treatment on the area of the POM ($F(2,14)=7.34$; $p<0.05$), such that SD birds on average possessed smaller POM (1.73 ± 0.09 mm^2) than Paired (3.22 ± 0.46 mm^2) and Alone birds (2.84 ± 0.23 mm^2) ($p<0.05$ for both comparisons), which did not differ from one another ($p>0.05$). Therefore, our photoperiodic treatments were successful in inducing a physiological condition characteristic of a breeding (Paired and Alone) and non-breeding (SD) state. Additionally, the endocrine state of the two LD groups was not significantly different based on the volume of their testes and on the measure of their brain exposure to T as reflected by the size of the POM.

Song rate and acoustic features

We first analyzed the song data by two-way ANOVAs with the three experimental groups as a between-subjects variable and the successive days as a repeated variable and did not find a significant interaction between these two factors for all of the measures studied ($p > 0.1$ for all interaction components of the Omnibus ANOVAs). Based on the lack of statistical effects associated with these interactions, we focused our analyses on the main effects of treatments on

all parameters for concision and clarity of data presentation. These data were thus analyzed by one-way ANOVAs focusing on the average/total measures across the entire experimental period. There was a significant effect of the different treatments on song rate ($F(2,14) = 6.52$; $p < 0.01$; Figure 2A). Males that were housed alone sang significantly more songs per hour as compared to SD ($p < 0.05$) and Paired ($p < 0.05$) birds. There was no significant difference in the song rate between Paired and SD ($p > 0.5$). There was also a significant effect of treatments on the average song duration ($F(2,14) = 7.25$; $p < 0.01$; Figure 2B). Males that were housed alone sang significantly longer songs compared to SD ($p < 0.01$) and Paired ($p < 0.05$) birds and there was no significant difference in song duration between these last two groups ($p = 0.65$).

Similarly we also detected significant treatment effects on the entropy variance ($F(2,14)=11.68$; $p<0.005$, Figure 1C) and energy ($F(2,14)=4.77$; $p<0.05$, Figures 2C-D). Compared to paired birds, males housed alone sang with more energy ($p<0.05$) and with a higher entropy variance ($p<0.005$). Compared to SD birds, however, birds housed alone did not differ in any of these acoustic measures ($p>0.17$ for all paired comparisons). SD birds sang with more entropy variance ($p<0.05$) than paired birds but did not differ from them with respect to the energy of their songs ($p>0.5$).

HVC area

There was a significant effect of treatment on the area of HVC at its largest extension ($F(2,14)=4.70$, $p<0.05$; Figure 3D), such that the Alone birds

possessed larger HVC than SD birds ($p<0.05$). Paired birds were indistinguishable from both Alone and SD birds.

DCX immunoreactive cells in HVC

We found a significant difference between groups in the number of DCX round cells in HVC ($F(2,14) = 4.71$, $p< 0.05$; Figure 3B). Male canaries that were paired with a female had significantly more DCX round cells compared to males housed alone ($p< 0.05$). There was no significant difference between paired and SD males ($p=0.18$) and between Alone and SD males ($p=0.49$). In contrast, we did not find a significant effect of treatments on the number of fusiform cells in HVC ($F(2,14) = 2.36$, $p= 0.13$; Figure 3C). There was also no effect of treatments on the number of round cells or fusiform cells in the region lateral (round: $F(2,14) = 0.65$, $P = 0.54$; fusiform: $F(2,14) = 1.03$, $P = 0.38$) or ventral (round: $F(2,14) = 1.17$, $P = 0.34$; fusiform: $F(2,14) = 0.1$, $P = 0.91$) to HVC.

Discussion

In this study, we assessed the effects of photoperiod and female presence on the neural and behavioral plasticity among males of a well-studied songbird species, the canary. We found that males housed alone in a breeding condition sing prolifically and with more acoustic complexity than males that were paired with a female. We also found that males housed with a female exhibited more DCX-ir round cells in HVC relative to birds on long days housed alone. These data suggest that the social context can regulate the incorporation of new

neurons into HVC as well as modulate features of song that are used by males to attract a potential mate.

These observations regarding the effects of social context on singing behavior are in line with the well-established hypothesis that song is used by males to attract a potential mate (Catchpole and Slater, 2003; Kroodsma and Byers, 1991). Males that were housed alone and in a breeding condition sang a larger number of songs with a greater length than birds that already possessed a female, supporting the idea that once a male has attracted a mate, it is economically favorable to reduce this form of courtship behavior (Sockman et al., 2005). Previous studies have tended not to test specifically the effects of a female on changes in song complexity. It has of course been shown that females do prefer more complex songs in a number of songbird species (Leitão, ten Cate, and Riebel, 2006). Other studies, however, have indicated the importance of emphasizing song features such as loudness to convey efficiently this reproductive signal to its receivers (H Brumm, 2004; Henrik Brumm and Slater, 2006; Henrik Brumm and Todt, 2002; Henrik Brumm, 2013), including females (Cynx and Gell, 2004). Our results indicate that male songbirds in search of a mate may sing with more complexity amid a loud signal to further increase the probability that they will attract a mate.

SD birds were similar to paired long day birds with respect to the number of songs, their duration, and their loudness. The presence of the female thus completely suppressed these song characteristics to the level observed under short day conditions. However SD birds were indistinguishable from long day

birds housed alone with respect song complexity. In canaries during the non-breeding season (i.e. between late summer and early winter) canaries are modifying their song by adding and deleting syllables until the song is eventually crystallized (Fernando Nottebohm, 1984; Voigt and Leitner, 2008). This must lead to increased variability in the song (i.e., increased entropy variance in the entire song) as birds are producing a variety of songs that they will use during the breeding season to attract a potential mate. Our entropy measure indeed quantifies variance in the entire song taking into account the changes from one syllable type to another. In the end, while long day birds housed alone and short day birds housed alone may be similar in this song measure, their reason for enhancing their song complexity may differ: in one case they are modifying their song before it eventually reaches its crystallized form (SD) while in the other they would be trying to attract a mate (Alone).

Previous work in canaries has shown that male canaries housed with a female exhibited enhanced HVC size relative to males housed with other males (Boseret et al., 2006). Here we found that males housed on long days with a female were not statistically different from birds housed alone on long days. It is, however, possible that, in the study by Boseret and colleagues (Boseret et al., 2006), males housed with females possessed larger HVC than those housed with a male because the presence of a male actually suppressed HVC size due to the stress associated with aggression, not because the presence of a female enhanced HVC size. Effects of stress and corticosterone on HVC size have

indeed been observed in a number of studies in several songbird species (zebra finches: Buchanan et al, 2004; song sparrows: Newman et al, 2010).

Although the paired birds had the same HVC size as the alone groups, they had significantly more DCX-ir cells in HVC despite the fact that they sang much less. This replicates a finding of the Boseret and colleagues studies in which males paired with a female sang less but had more DCX-ir cells in HVC than males paired with another male (Boseret et al., 2006; Balthazart et al., 2008). These data lead to two important conclusions. Firstly they indicate that the size of HVC does not necessarily correlate with the number of DCX-ir cells. Such a correlation would be expected and was observed at some time points when the size of the nucleus and the rate of neurogenesis are dynamically changing (e.g. See Balthazart et al., 2008; Yamamura et al., 2011). It is also conceivable that changes in these two variables are not necessarily synchronous: after a given stimulation, the size may change later than the numbers of DCX-positive cells and this would obviously result at specific time points in discrepancies between these two variables as observed here. This prediction is in line with previous studies showing that the dramatic seasonal increase in the size of HVC does not occur until several days after the incorporation of new neurons (Alvarez-Buylla et al., 1992; John R Kirn, 2010; Fernando Nottebohm, 1984).

Secondly the present data demonstrate that the density of DCX-ir cells in HVC is controlled by a variety of factors acting in a semi-independent manner and including exposure to T, singing activity and presence or absence of a female partner. Previous studies in canaries revealed that the production of song

by itself leads to enhanced expression of trophic factors such as brain derived neurotrophic factor (BDNF) in HVC, which causes an increase in the incorporation of new neurons in this brain region (Alvarez-Borda and Nottebohm, 2002; Li et al., 2000). Therefore, one may have expected to observe a larger number of DCX-ir cells in birds from the alone group (who sang at a high rate) as compared to the paired birds (that were singing very rarely), but the opposite result was observed. It is important to note in this context that, regardless of the social context (Paired vs. Alone), birds housed on long days possessed similarly enlarged testis size and POM nuclei of similar sizes, suggesting that both were in a similar breeding condition and exposed to similarly high circulating T concentrations. It is therefore unlikely (although cannot be completely excluded) that differences in circulating T led to the observed differences in DCX-ir cells, especially given that HVC size (a T-dependent variable) was equal in both the Paired and Alone groups. The large number of DCX-ir cells in the Paired group thus seems to relate specifically to the presence of the female independently of the singing activity and of the endocrine (plasma T) condition. It remains nevertheless possible that long day birds that were housed alone may have experienced some form of stress, which could have reduced the number of DCX-immunoreactive round cells in HVC (see above and Buchanan et al, 2004; Newman et al, 2010).

Overall, the results of this study support the importance of one of the well-known proposed functions of birdsong as applying to canaries, mate attraction, and suggest that male songbirds emphasize specific features of song to

accomplish this. These data also suggest that the social context may have a very substantial effect on neurogenesis, and in some contexts at least possibly more important than the production of song. This increased number of round DCX-ir neurons present in HVC at 14 days after the placement in experimental conditions provides an integrated view of the dynamic changes in neurogenesis that took place during this period. It does not however allow us to determine with certainty whether these changes are linked to modifications in cell proliferation, migration, recruitment, differentiation and survival. It provides a result that reflects the sum of these measures. By counting separately the fusiform (presumably still migrating) and round (currently differentiating) DCX-ir cells we obtain, however, a view of two cell populations that are at different stages of their ontogeny and were thus in all likelihood born at different times before brain collection. This suggests that the presence of a female rapidly affected neurogenesis so that 14 days later, there was an effect on cells that has already been recruited and had initiated their differentiation in HVC. Future studies combining BrdU injections and DCX immunohistochemistry should now be performed to dissect the interesting phenomenon.

The adaptive consequence of neurogenesis in HVC in paired birds remains to be investigated, but the present results suggest that if paired birds do sing, such as during later stages of the reproductive cycle (Hinde and Matthews, 1957; Hinde and Steel, 1976; Alward *et al.*, 2013), they will have an increased capacity for producing attractive, high quality song (Leitner and Catchpole, 2004).

Figure Legend

Figure 1- A) Schematic of HVC and its main synaptic targets in RA and Area X. B) Effects of treatment on breeding condition as measured via the volume of the testis. Bar graphs are means ± SEM. Bars with a different letter are significantly different for $p \leq 0.05$, bars with a same letter are not different. C). Representative songs from each treatment group.

Figure 2- Effects of treatment on song output (A. Song rate (number per hour); B. Mean song duration) and its acoustic features (C. Energy; D. Entropy variance, see text for additional explanations) in male canaries. Bar graphs are means ± SEM. Bars with a different letter are significantly different for $p \leq 0.05$, bars with a same letter are not different.

Figure 3- The effects of treatment on DCX immunoreactivity in HVC and on HVC area. A) Representative photomicrograph depicting DCX round (left panel) and fusiform (right panel) cells. Magnification bar=50 μm B) Effects of treatment on the number DCX round cells per mm^2 in HVC. C) Effects of treatment on DCX fusiform cells per mm^2 in HVC. D) Effects of treatment on HVC area at its largest extension. Bar graphs are means ± SEM. Bars with a different letter are significantly different for $p \leq 0.05$, bars with a same letter are not different.

Figure 1

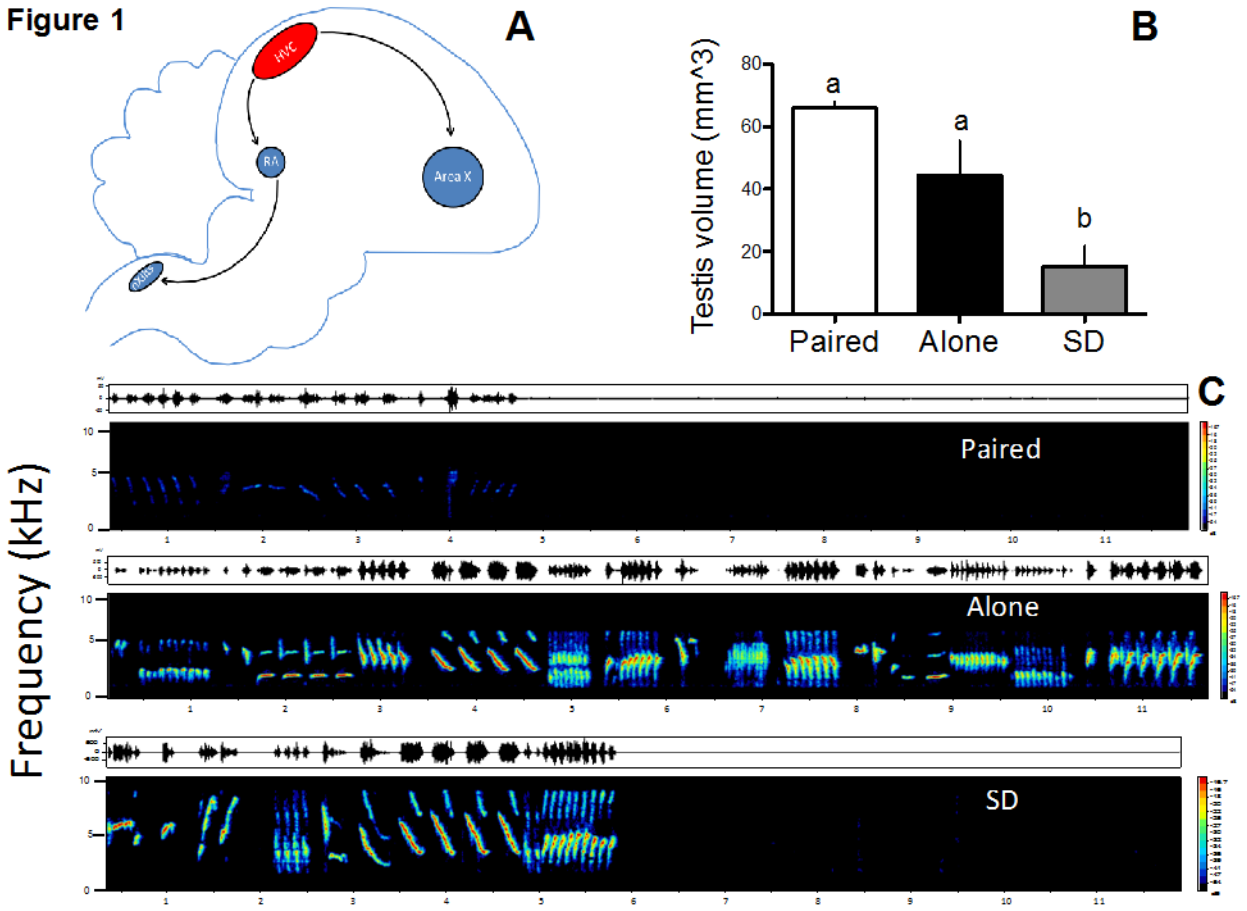

A
HVC
RA
Area X
LMAN
B
Testis volume (mm^3)
80
60
40
20
0
a
a
b
Paired
Alone
SD
C
Frequency (kHz)
Paired
Alone
SD

Figure 2

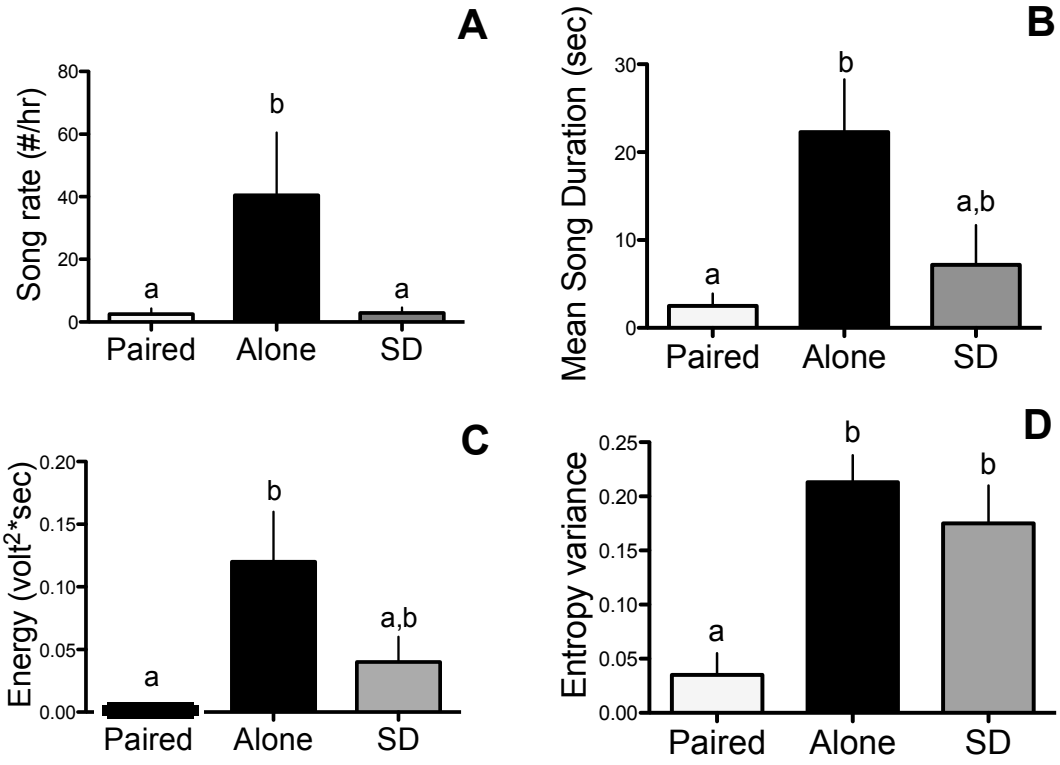
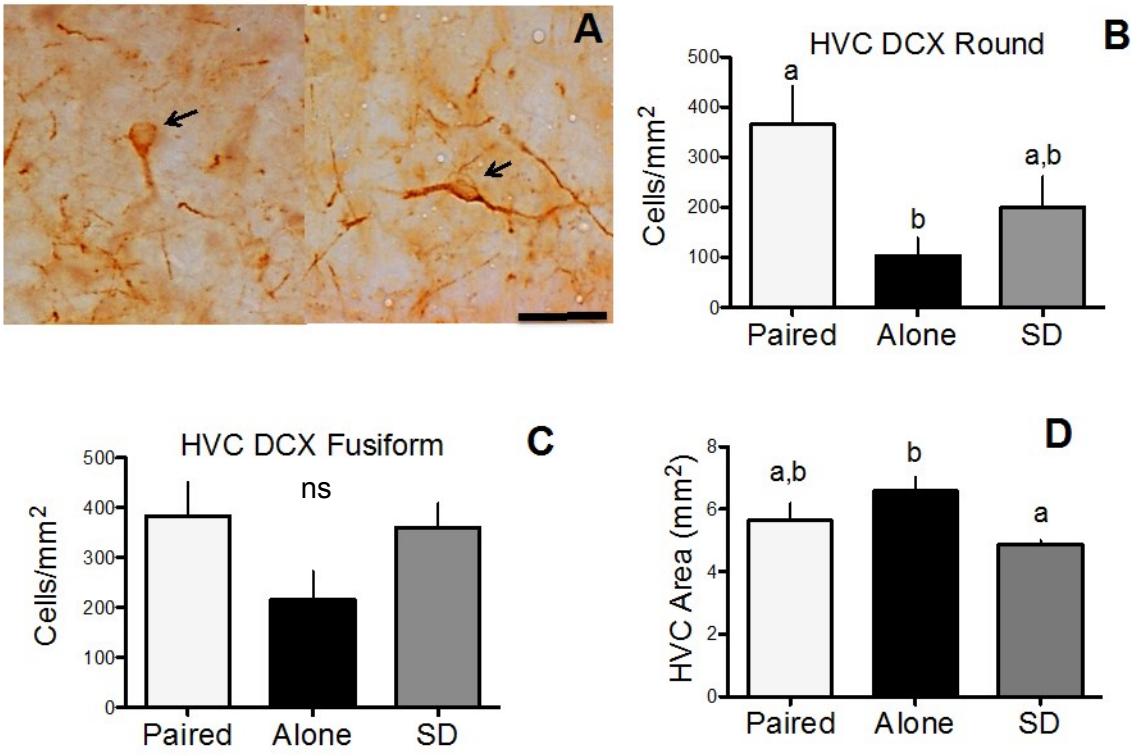

A
Song rate (#/hr)
80
60
40
20
0
a
b
a
Paired
Alone
SD
B
Mean Song Duration (sec)
30
20
10
0
a
b
a,b
Paired
Alone
SD
C
Energy (volt²*sec)
0.20
0.15
0.10
0.05
0.00
a
b
a,b
Paired
Alone
SD
D
Entropy variance
0.25
0.20
0.15
0.10
0.05
0.00
a
b
b
Paired
Alone
SD

Figure 3


A
B
HVC DCX Round
Cells/mm2
500
400
300
200
100
0
a
b
a,b
Paired
Alone
SD
C
HVC DCX Fusiform
ns
Cells/mm2
500
400
300
200
100
0
Paired
Alone
SD
D
HVC Area (mm2)
8
6
4
2
0
a,b
b
a
Paired
Alone
SD

Chapter 3: Investigating the effects of bicalutamide, a peripherally selective androgen receptor antagonist, on song in canaries (*Serinus canaria*)

Introduction

Seasonal changes in brain and behavior are often mediated via the actions of gonadal steroid hormones (Ball and Balthazart, 2002; Tramontin and Brenowitz, 2000; Wingfield and Farner, 1993). As reviewed in the Introduction, there are multiple sites of steroid action throughout the songbird brain (Ball et al., 2002; Bernard et al., 1999; Metzdorf, Gahr, and Fusani 1999). Given this widespread distribution of sex-steroid hormone receptors and the complex nature of the learning and generation of songbird vocalizations, many have focused on the central regulation of birdsong. However, the syrinx, the vocal production organ, also expresses a high number of androgen receptors (Lieberburg and Nottebohm, 1979) and has been studied extensively in terms of its role in the generation of avian vocalizations in non-songbirds as well as songbirds (Casey and Gaunt, 1985; Gaunt and Gaunt 1977; Goller and Suthers, 1996; Larsen, Goller, and Leeuwen, 2006; Suthers, 2004; Wild, 1997). Moreover, Nottebohm and others have investigated how T can regulate both physiological and morphological characteristics of the syrinx. For instance, castration reduces the mass of the syrinx as well as the activity of the two enzymes, chloline acetyltransferase and acetylcholinesterase in canaries (*Serinus canaria*) and zebra finches (*Taeniopygia guttata*) (Brenowitz and Lent, 2002; Luine et al., 1980).

The current experiment will delve into a specific way T might regulate song in canaries by investigating how bicalutamide (BICAL), an androgen receptor antagonist that does not cross the blood-brain barrier, affects song in canaries (Furr and Tucker, 1996). Subsequent studies will focus on the role played by androgens acting on brain sites relevant to song control.

Many studies have assessed how hormones such as T affect song in songbirds. For instance, castration drastically reduces song output based on studies in several species (Alvarez-Borda and Nottebohm, 2002; Alward et al., 2013; Arnold, 1975; Sartor et al., 2005) and song during the breeding season, when circulating levels of T are high in temperate-zone species including white-crowned sparrows and canaries, is more stereotyped and of longer duration compared to the non-breeding season, when circulating levels of T are low (Smith et al., 1997; Voigt and Leitner, 2008).

However, the question of the effects of T action specifically at the level of the syrinx in the regulation of song has been addressed only recently. For instance, in a non-songbird, the golden-collared manakin (*Manacus vitellinus*), treatment with BICAL, an androgen receptor blocker that does not cross the blood-brain barrier (Furr and Tucker, 1996), caused changes in the temporal and pitch characteristics of their *chee-poo* call (Fuxjager, Heston, and Schlinger, 2014). In songbirds, there is no direct evidence for the role of T at the syrinx in the regulation of song.

In the current study, male canaries were administered BICAL to test the role of androgen action at the syrinx in the regulation of song. As BICAL does not

cross the blood-brain barrier, and the syrinx is the most behaviorally relevant androgen-receptor dense organ in the periphery in regards to birdsong, this is currently one of the most useful approaches currently available to study the specific role that androgen action at the syrinx plays in the regulation of birdsong. Canaries are especially suitable for an investigation such as this, given extensive studies on their song including detailed investigations into the syringeal mechanisms involved in generating vocalizations called trills. Trills are a key part of canary song and include a special trill type that is a physiologically demanding, highly sexually-relevant vocal signal called “special syllables” or “sexy syllables” by some authors (Podos, 1997; Suthers, Vallet, and Kreutzer, 2012; Suthers, et al., 2004; Vallet et al., 1998; Vallet and Kreutzer, 1995).

Methods

Materials and Methods

Animals and pre-experimental manipulations. Canaries (*Serinus canaria*) of the Border strain were used for this study. Male and female canaries were obtained from a local breeder (Maryland Exotic Birds). Upon entry into the lab birds were placed on a short day photoperiod (8L:16D) for six weeks to induce photosensitivity (Dawson et al., 2001; Nicholls and Storey, 1977). Birds were housed in groups in mixed-sex housing.

Implantation of BICAL. Silastic implants of BICAL were used to deliver BICAL in this experiment. Birds were placed on their right side and anesthetized using %2.5 isoflurane gas. Implants were inserted that were10 millimeters in length with 8 mm of BICAL exposed to the silastic surface. There are at present no

published research papers employing Silastic implants to deliver BICAL. However, given BICAL's similar lipophilicity to antiandrogens such as flutamide, which have been used at similar Silastic implant lengths, this length of BICAL was selected (Alward et al., 2013; Robertson, et al., 2014). Control implants were also 10 mm in length but were left empty. Incisions were made above the shoulder and the implants inserted subcutaneously; incisions were sealed using Vetbond. 7 birds were implanted with BICAL and 7 were implanted with blanks.

Song recording and analysis. On the day of implantation, birds were placed into individual, sound-attenuated recording chambers as described in chapter 2 housed on long days (14L:10D) to induce a state of photostimulation characteristic of the breeding season (Dawson et al., 2001; Nicholls and Storey, 1977). Birds remained in these chambers for 21 consecutive days. Song recording and analysis settings (e.g., filter settings, sampling rate, etc) were identical to those in chapter 2. Each day video and audio recordings were made from 800h to 1030h (lights on at 800 h). We quantified the song features listed in the Appendix on days 1,3,5,7,9,12,16,18, and 21 using the sound analysis program Avisoft (Alward et al., 2013). Songs were defined as vocalizations being longer than or equal to 1 second in duration and separated by 500 milliseconds of silence (Alward et al., 2013; Voigt and Leitner, 2008).

We also quantified song stereotypy (Meitzen et al., 2007). Song stereotypy indicates how similar certain features of song are across song renditions. Song stereotypy was determined by calculating the coefficient of variation (CV=(SD/AVG)*100)) using the standard deviations of song acoustic features

(SD) described in the Appendix and dividing this by the average (AVG) across the same values used to calculate the SD. CV is an inverse measure of song stereotypy; therefore we used the inverse of CV multiplied by 100 to facilitate data presentation.

We were also interested in analyzing a particular feature of canary song, called trills. In canaries, like many other songbirds, there is a strong evolutionary pressure for the production of trills with high repetition rate that are physiologically difficult to produce (Podos, 1997) and extensive research has described the complex morphological changes the syrinx undergoes in real time for quality trills to be produced (Goller and Suthers, 1996; Suthers, Vallet, and Kreutzer, 2012; Suthers et al., 2004; Suthers, 2004). Given the effects of T on acetylcholine activity and thus neural firing characteristics of the syrinx (Luine, Nottebohm, and Harding, 1980) and the strong sexual selection postulated to have shaped the production of trills (Podos 1997), we hypothesized that BICAL would have a substantial effect on these types of vocalizations. Recordings from days 1, 3, 5, 7, 9 12, and 21 were assessed for these trills. On each of these days, we looked specifically within song bouts and analyzed trills within a bout of continuous singing of five minutes. A trill is defined as a rapidly sung sequence of repeated notes (Podos, 1997). Using the Avisoft program we were able to save images of each trill and quantify features of all of these trills; we quantified the same features for trills as we did for whole songs. Images of each trill type were saved into Powerpoint and all acoustic parameters were analyzed per each trill and collapsed into overall trill values.

Brain, syrinx, and blood collection. 21 days after treatment initiation, birds were deeply anesthetized (4% Isoflurane), weighed, and their brain was extracted and fixed in acrolein after collecting blood from the trunk region into 1.5 ml centrifuge tubes. The syrinx was then extracted and placed on dry ice for ≥ 5 minutes before it was weighed. Blood was spun down at 8,000 rpm for 6 minutes and serum was collected and stored in a freezer at -20° C. Brains were agitated in acrolein for 2 hours, then washed for 15 minutes X four times in phosphate buffered saline (PBS) and placed in sucrose (30% solution in PBS) over night until they sank to the bottom of the vial. After this cryoprotection by sucrose, brains were flash frozen in dry ice for 5 minutes, and then placed into a -70° C freezer.

Brain and serum analyses. Brains were sectioned from the olfactory bulb to the brainstem in the transverse plane using a cryostat at 30 microns into four series of sections that were stored in cryoprotectant. These four series were placed into a -20° C freezer. One series was later mounted on gelatin-coated slides and exposed to air for a day. Then, mounted sections were exposed to a standard Nissl staining procedure and coverslipped using Permount (Fisher Scientific). Concentrations of serum T were determined using an enzyme-linked immunosorbent assay ELISA that has been used previously in our lab for canaries (Madison et al., 2015; Enzo Life Sciences, Testosterone ELISA kit, catalog #ADI-900-065, Plymouth, PA).

SCS and POM volume reconstruction. Photomicrographs of HVC, RA, and nXIIts were taken at 2.5x magnification in the Nissl-stained sections using an axiocam attached to a Zeiss axioskop. As others have done in previous studies, the area of each nucleus was determined in both hemispheres of each section where it appeared using NIH Image J and volumes were determined by multiplying areas by the section thickness, summing these values, and then multiplying this value by 4, since only every 4th section was Nissl stained (Alward et al., 2013; DeVoogd et al., 1991; Sartor et al., 2005). For HVC and RA, these nuclei were quantified as our lab has shown before (Alward et al., 2013); for nXIIts, we used similar methods based on work by DeVoogd et al. (1991). All of these song control nuclei express AR and the nucleus volume size tends to be positively correlated with circulating concentrations of T (e.g., Brenowitz and Lent, 2002; DeVoogd et al., 1991; Sartor et al., 2005). In addition to quantifying the SCS volumes to test BICAL's efficacy, we also wanted to test the hypothesis proposed by Fuxjager et al (2014) and Larson et al (2013) that decreases in neural activity at an effector site (e.g., the syrinx) may retrogradely affect neural plasticity in the regions that provide input. We also quantified the volume of the POM as it extends from its intermediate position (the DSV extends across the basal level of the brain after the TSM split disappears) to its caudal level (the anterior commissure extends across the midline fully). The volume of the POM is highly sensitive to the concentrations of T (Alward et a;., 2013; Charlier et al., 2008; Riters et al., 2000), with T correlating positively with POM volume. Thus, the volume of the POM is a sensitive marker of T present in the cerebrospinal

fluid (CSF) and general circulation, and was thus used as a proxy for the efficacy of BICAL as a peripherally-selective AR antagonist.

Statistical Analyses. A total of four birds did not sing at all during the experiment (BICAL, n=2; EMPTY, n=2). Thus, these birds were excluded from all song analysis (final N: BICAL, n=5; EMPTY, n=5) but were included in the assessment of BICAL's effects on all physiological measures.

For days 9,12,16, 18, and 21 all 10 birds sang. From days 1-7, however, song rate between birds, regardless of treatment, was much more variable, with anywhere between 1 and 4 birds per treatment group not singing. On day 1, 1 BICAL bird sang and 2 EMPTY birds sang. This is consistent with previous work on the time needed for long days to induce singing in canaries (Alward et al., 2013). For this reason, we analyzed song from days 3-9 in time blocks; specifically, we used a time block for days 3-5 and days 7-9. During time block 3-5, all birds from the BICAL group were singing (n=5) while 4 out of the 5 EMPTY birds were singing. For the time block 7-9, all birds from both groups sang. Hence, for the analysis of song a mixed-design design using ANOVAs was used for days 7-9, day 12, day 16, day 18, and day 21 with treatment as the between-subjects factor. A t-test was used on days 3-5 to assess an effect of treatment. A similar approach was used for trills, but days 7-9, 12, and 21 were included in the mixed-design and a t-test was used on days 3-5 to determine effects of treatment.

Following a significant omnibus ANOVA, Scheffe's contrasts were used as post-hoc tests. However, it is plausible that the effects of BICAL on song may be

minimal and/or short-lasting. This prediction stems from observations made by Brainard and colleagues (Sakata and Brainard, 2006; Sober and Brainard, 2008) that birds can adaptively modify their song when song is perturbed, and this modification can occur relatively rapidly, ranging from 1-3 days following a perturbation. Since BICAL is peripherally-selective, androgenic action in the brain is intact; hence, any androgen-driven song correction mechanisms may still be intact. Therefore, to decrease our chances of not detecting an effect that could be washed out in an omnibus ANOVA, in some cases we conducted t-tests for variables where it seemed an effect might be present on certain days. To minimize type I error, for these comparisons we used a corrected alpha value of 0.02 (Sidak correction for two comparisons). The number of these t-tests in these cases never exceeded two.

Results

BICAL acts in a peripherally selective manner. Birds treated with BICAL had smaller syrinxes compared to birds treated with an empty implant (Figure 1; t-test, $p<0.05$). There was no effect of BICAL on the volumes of POM, HVC, RA, nXIIts, or circulating concentrations of T (Figure 1; $p>0.2$ for all comparisons). Therefore, BICAL appears to act selectively in the periphery and not centrally (Alward et al., 2013, 2014; Fuxjager et al., 2014; Fuxjager, 2013; Meitzen et al., 2007).

BICAL did not affect singing activity but increased song duration and disrupted song acoustic measures in an acute manner. As expected, treatment with BICAL did not affect singing activity (Figure 2; song rate and %

time spent singing, ($p \geq 0.17$ for all sources of variation in the ANOVA). There was a tendency for an effect of treatment on song duration (Treatment, $p=0.08$; treatment*day and day, $p \geq 0.39$). However, on days 3-5 and 7-9, BICAL birds sang longer songs than EMPTY birds (t-tests, $p<0.05$ and $p=0.02$,respectively). The overall ANOVA for song energy revealed non-significant effects ($p \geq 0.25$ for all sources of variation in the ANOVA). However, on days 7-9 BICAL birds sang louder songs than EMPTY birds (t-test, $p=0.02$). The overall ANOVA for entropy variance did not yield any significant effects ($p \geq 0.22$ for all sources of variation in the ANOVA). On day 16 EMPTY birds sang with more entropy variance than BICAL birds, but this did not reach significance based on the corrected alpha level of 0.02 for individual t-tests ($p=0.04$). For song maximum frequency variance, there was a significant interaction and a significant effect of day ($p<0.05$ for both) but not an overall effect of treatment ($p=0.11$). Post-hoc contrasts revealed EMPTY birds sang with more maximum frequency variance than BICAL birds on days 12 and 21 but these groups were not different on any other days ($p<0.05$ for both comparisons). Treatment with BICAL did not affect the fundamental frequency of song or any of the stereotypy features of song ($p \geq 0.25$ for all sources of variation in the ANOVA).

BICAL perturbed multiple trill features. Treatment significantly affected the duration of trills (Figure 4; Treatment, $p<0.05$; treatment*day, $p=0.13$; day, $p=0.52$). On all days BICAL birds sang longer trills than EMPTY birds (post-hoc contrasts, $p<0.05$; days 3-5, t-test, $p<0.05$). There was also an effect of treatment on syllable repetition rate within trills (treatment, $p<0.05$; treatment*day, $p=0.65$;

day, $p<0.05$), wherein EMPTY birds had higher syllable repetition rate than BICAL birds on days 3-5 ($p<0.05$) and 7-9 and day 21 ($p<0.05$). Treatment also affected the pitch of trills (Treatment, $p<0.05$; treatment*day, $p=0.75$; day, $p=0.99$). Specifically, on day 21 BICAL birds sang trills that were higher in pitch than EMPTY birds. Moreover, BICAL enhanced the energy of trills (Treatment, $p<0.05$; treatment*day, $p=0.11$; day, $p=0.21$) such that on days 7-9, 12, and 21, BICAL birds sang louder songs ($p<0.05$). On days 3-5 there was a tendency for BICAL birds to sing louder songs ($p=0.06$). There was no overall effect of treatment on fundamental frequency variance (Treatment, $p=0.07$; treatment*day, $p=0.96$; day, $p=0.78$); however, an individual t-test on day 21 suggests a tendency for EMPTY birds to sing with more fundamental frequency variance ($p=0.07$). Lastly, treatment affected the entropy variance stereotypy of song (Treatment, $p<0.05$; treatment*day, $p=0.09$; day, $p=0.18$), such that on days 7-9 BICAL birds sang more stereotypic trills compared to EMPTY birds ($p<0.05$).

Discussion

In this study we tested the effects of BICAL, an androgen receptor blocker that does not cross the blood-brain barrier (Furr and Tucker 1996), on song in canaries. Our goal was to investigate how T acting solely in the periphery, with particular relevance towards the actions of T at the syrinx, plays a role in regulating birdsong. As expected, BICAL caused no changes in the volumes of T-sensitive brain regions and motivational measures of song yet decreased the mass of the syrinx, indicating that BICAL acts in a peripherally-selective manner. Blocking AR peripherally using BICAL led to perturbations of song and trill

acoustic structure. Overall, activation of AR in the periphery, presumably at the syrinx, is involved in maintaining the overall acoustic structure of songs and trills, especially relevant social signals that have undergone strong sexual selection (Podos, 1997).

Our results suggest that activation of AR at the syrinx sustain the acoustic complexity of songs and trills without affecting any motivational measures of song. There was an effect of BICAL on trill stereotypy, but in the direction opposite of what was predicted for the effects of T on song stereotypy. Based on previous studies, however, blocking AR at the syrinx may cause trills to be *super* stereotypic, and may have actually reduced their effectiveness as a social signal. Indeed, work by Pasteau and colleagues (Pasteau et al., 2004, 2012) have shown that trills with more variation are more effective at sexually stimulating females. This interpretation is in line with our results here showing that BICAL-treated birds produced trills with slower repetition rate and higher acoustic frequency, as both of these perturbations decrease the overall effectiveness of trills in attracting a mate (Pasteau et al., 2007; Podos 1997).

Interestingly, birds treated with BICAL produced both songs and trills that were longer than those produced by control birds. This result parallels observations made by Fuxjager et al. (2014), who showed that golden-collared manakins (*Manacus vitellinus*), a sub-oscine, produced sexually-relevant calls that were longer when treated with BICAL. These authors posited that this increase in call duration might have been due to retrograde decreases in neural functions in nXIIts. In our study, we did not observe a decrease in the volume of nXIIts in

BICAL-treated birds. This seems to go against the retrograde hypothesis but it certainly does not preclude it as these effects could have been mediated by nXIIts in the absence of a major morphological change occurring. Lastly, it is intriguing to consider the duration increase in light of the observation that BICAL-treated birds produce trills with slower repetition rate. This slower repetition rate may have contributed to the overall increase in duration in trills and perhaps songs.

In contrast, the increase in song and trill duration may not be an effect of BICAL *per se,* but instead a consequence of perturbed song. Indeed, in the context of the increase in the energy of songs and trills, these changes are reminiscent of an animal that has entered a state of an enhanced attempt to closer match its current song production to its auditory template (Brumm and Todt 2002; Brumm and Zollinger 2011; Kuhl 2003). For instance, effects like these have been observed in many animals, including humans and songbirds, when an animal experiences a decrease in the effectiveness of its vocal signal (e.g., caused by environmental noise). For the present results, the perturbed song would be the environmental noise, which would lead the bird to alter its vocal signal to restore its acoustic characteristics. Notably, as observed by Rouse and Ball (2015), canaries produce songs with greater variation in amplitude during their sensorimotor phase of song learning, a phase of song learning marked by vocal exploration hypothesized to be an attempt to match the bird's own song template, which it stored during early development. This observation, that perturbations in one's own vocal signal, and thus a perturbed

feedback relative to ones own template for learned vocalizations, would be in line with what has been observed in deafened humans (Lane and Tranel, 1971) and budgerigars (*Melopsittacus undulates*; Heaton et al., 1997) and zebra finches that have had their auditory feedback perturbed (Cynx and Rad 2001). Hence, the disrupted acoustic structure of song and trills caused by BICAL may have caused the bird to enter a state of adaptive vocal modifications to more closely match its song to its stored song template. Indeed, Brainard and colleagues (Brainard and Doupe, 2000a; Sober and Brainard, 2009) have shown that songbirds that have experienced even small levels of perturbed auditory feedback will attempt to correct the perceived vocal production error, even within one to three days. In fact, this ability of songbirds to adaptively modify their vocalizations when they are perturbed may explain why some of the effects of BICAL on song were relatively short-lasting and/or rare.

The current results provide support for the idea that sex steroid hormones like T must act at multiple levels to coordinate distinct features of a single behavior into a functional response (Arnold, 1981). Previous work has shown, for instance, that T in the POM is sufficient to activate the motivational measures of song (Alward et al., 2013; Chapter 4, current thesis) while T in the HVC is required for activate the stereotypy features of song and may partially regulate the complexity features of song (Chapter 5; Meitzen et al., 2007). Based on these observations and the role of T in inducing normal neural activity in the syrinx, it is not surprising that BICAL caused disruptions in the acoustic structure of song and trills.

From an evolutionary perspective, the effects of BICAL on trills are quite intriguing. Indeed, blocking peripheral AR, presumably via AR at the syrinx, caused birds to produce trills that were slower in repetition rate and of higher acoustic frequency, both perturbations that decrease the effectiveness of trills in terms of mate attraction (Pasteau et al, 2007; Podos, 1997). Combined with the ideas put forth by Podos (1997), we postulate that AR at the syrinx may be a critical site for sexual selection to act in the shaping of birdsong, especially birdsong in which trills are used. It has been observed in the past that the effects of T on male courtship and copulatory behaviors often involve its conversation to an estrogen in the CNS but it conversion to an androgen in the periphery (Ball and Balthazart, 2002). Our results here are consistent with that general notion.

Figure Legend:

Figure 1-The effects of BICAL versus an empty implant on multiple measures of BICAL efficacy. The mass of the syrinx is an indicator of the peripheral effects of BICAL while all the effects of BICAL on T concentration, POM volume, RA volume, nXIIts volume, and HVC volume are proxies for the central actions of androgens. Bars represent the Mean ±Standard error of the mean. Asterisks indicate a significant difference at P≤0.05.

Figure 2-The effects of birds treated with BICAL versus birds treated with an empty implant on song over time. Graphs depict various motivational measures of song (song rate, time spent singing, and duration) and a number of acoustic features that are described in the appendix. Bars represent the Mean ±Standard error of the mean. Asterisks indicate a significant difference at p≤0.05 or p≤0.05 for duration and energy.# denotes p=0.04 versus an alpha level of 0.02.

Figure 3- The effects of birds treated with BICAL versus birds treated with an empty implant on trills over time. Graphs depict the effects of BICAL on number of acoustic features and trill stereotypy, which are described in the appendix. Bars represent the Mean ±Standard error of the mean. Asterisks indicate a significant difference at P≤0.05. .# denotes p=0.07 versus an alpha level of 0.02.

Figures

Figure 1

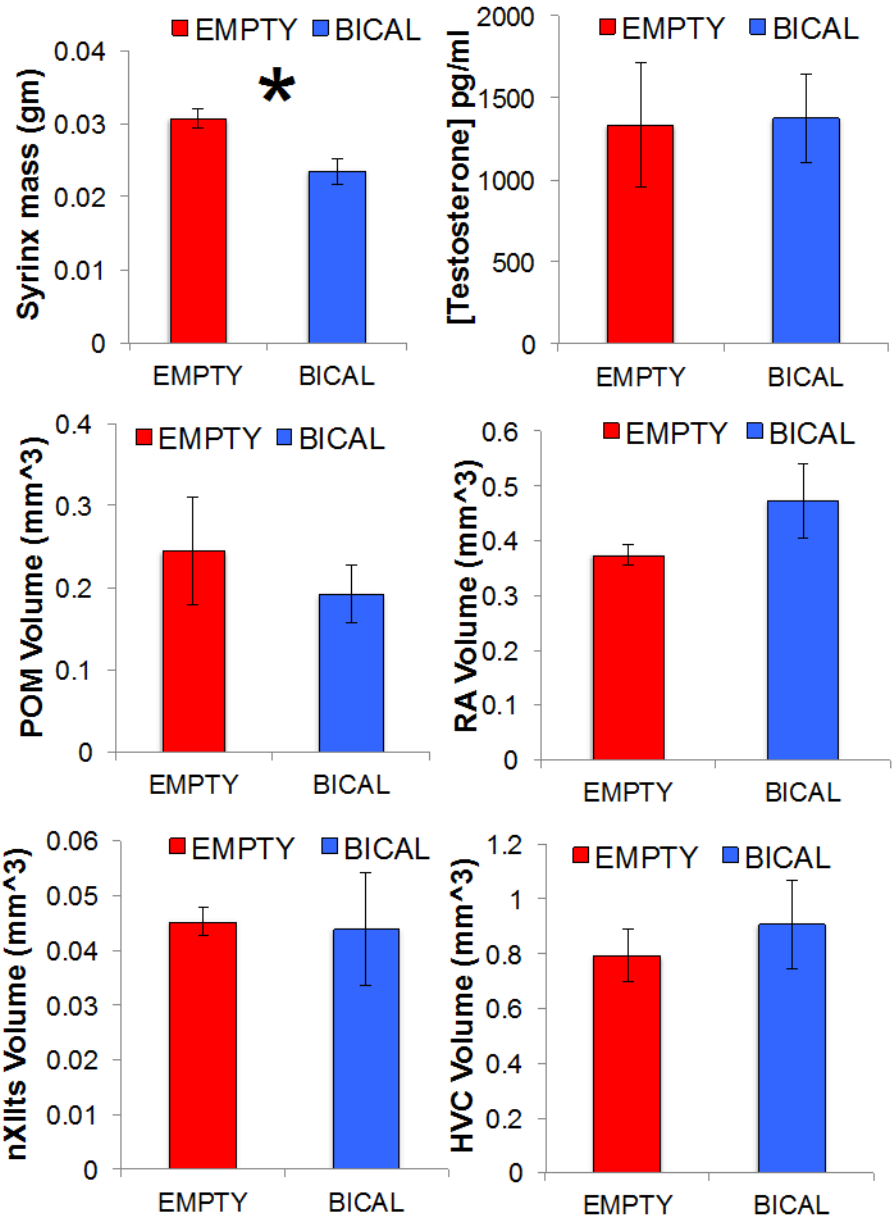


Figure 2

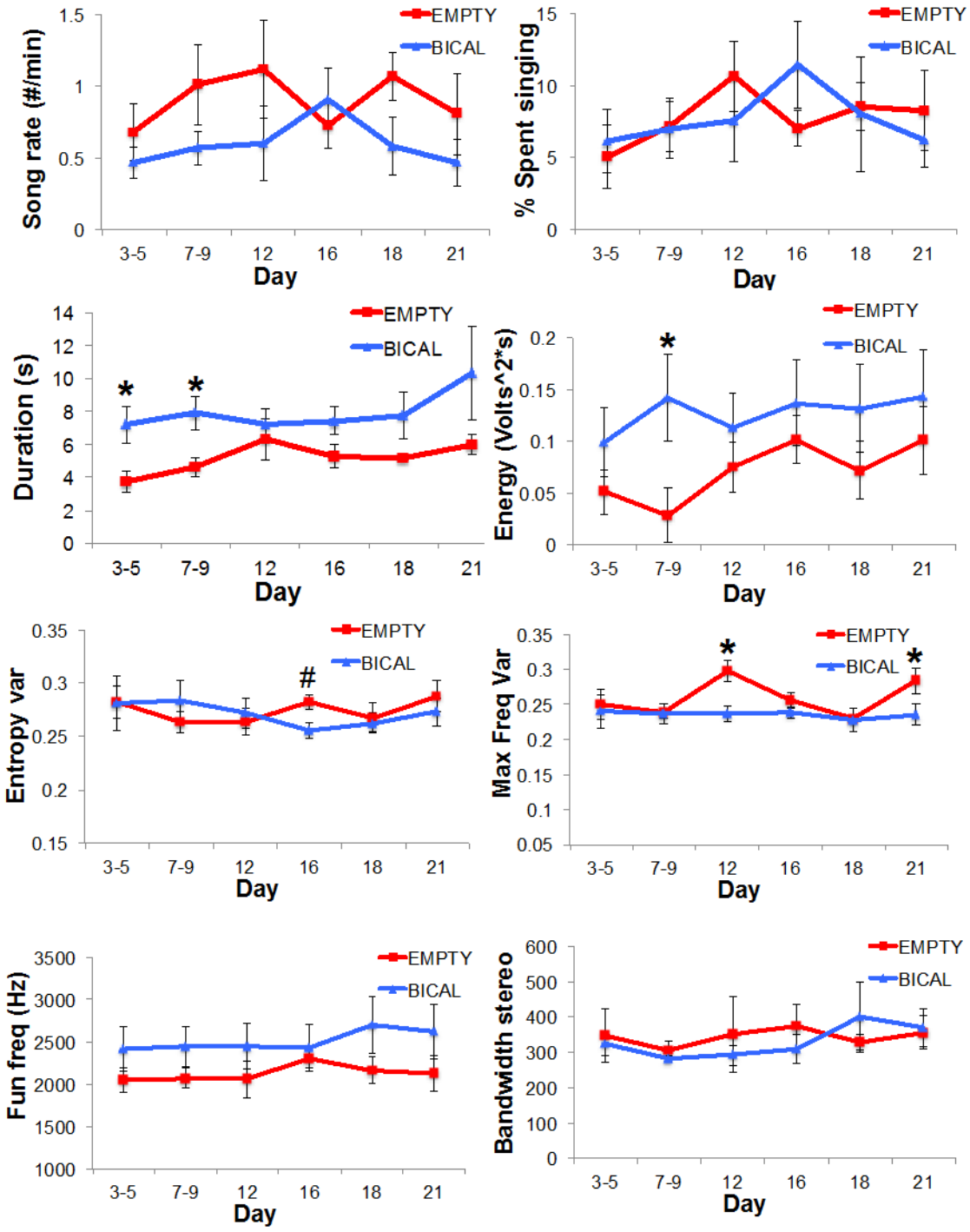
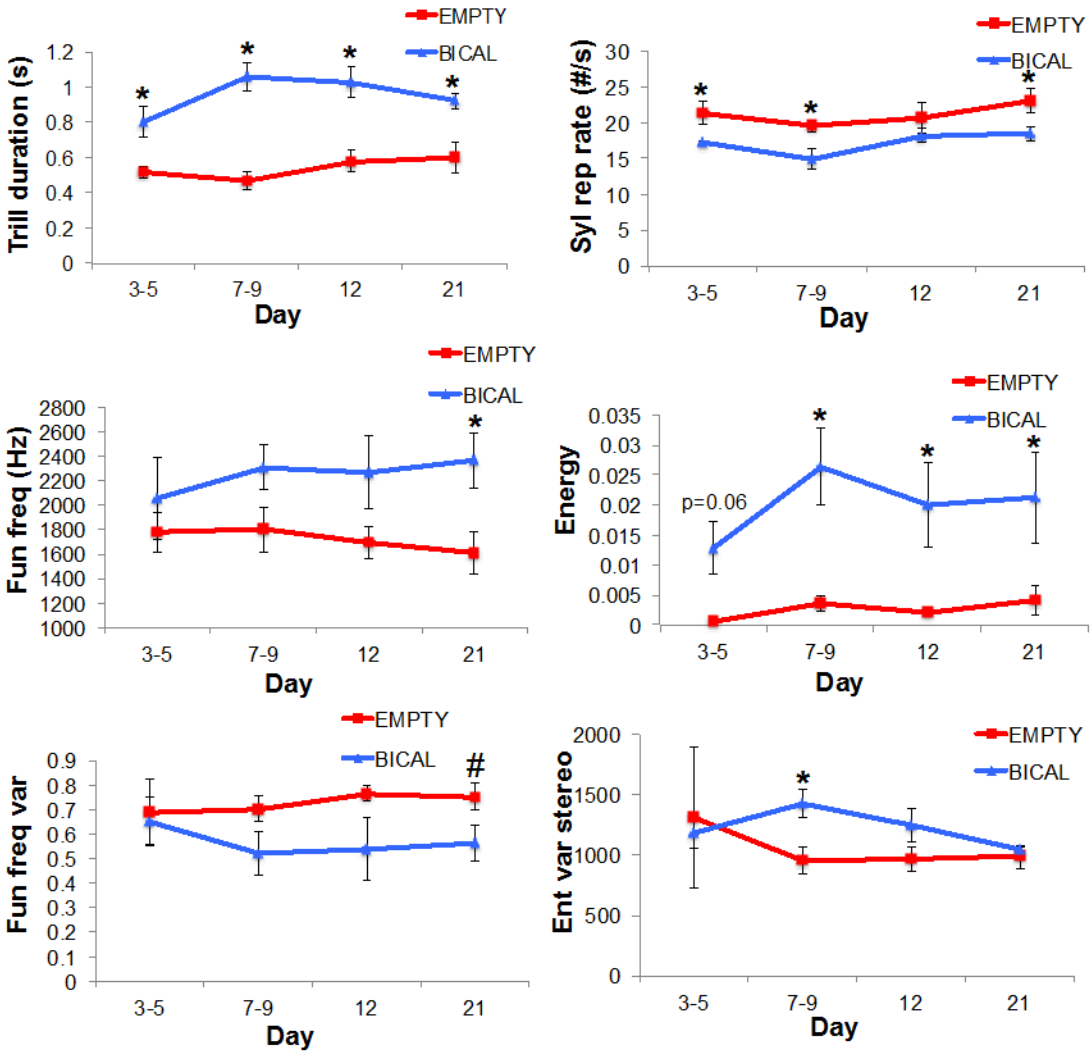

Song rate (#/min)
EMPTY
BICAL
Day
% Spent singing
Duration (s)
Energy (Volts^2*s)
Entropy var
Max Freq Var
Fun freq (Hz)
Bandwidth stereo
3-5
7-9
12
16
18
21
*
#

Figure 3


EMPTY
BICAL
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Syl rep rate (#/s)
Fun freq (Hz)
Energy
p=0.06
Fun freq var
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Day
3-5
7-9
12
21
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#

Chapter 4: Differential effects of global versus local testosterone on singing behavior and its underlying neural substrate

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Title: **Differential effects of global versus local testosterone on singing behavior and its underlying neural substrate.**

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Abstract

Steroid hormones regulate multiple but distinct aspects of social behaviors. T has multiple effects on learned courtship song in that it regulates both the motivation to sing in a particular social context as well as the quality of song produced. The neural substrate(s) where T acts to regulate the motivation to sing as opposed to other aspects of song has not been definitively characterized. We show here that T implants in the preoptic medial nucleus (POM) of castrated male canaries (*Serinus canaria*) increases song rate but does not enhance acoustic features such as song stereotypy as compared to birds receiving peripheral T that can act globally throughout the brain. Strikingly, T action in the POM increased song control nuclei volume, consistent with the hypothesis that singing activity induces neuroplasticity in the song control system independent of T acting in these nuclei. When presented with a female canary, POM-T birds copulated at a rate comparable to birds receiving systemic T but produced fewer calls and songs in her presence. Thus POM is a key site where T acts to activate copulation and increase song rate, an appetitive sexual behavior in songbirds but T action in other areas of the brain or periphery (e.g., HVC, dopaminergic cell groups or the syrinx) is required to enhance the quality of song (i.e. stereotypy) as well as regulate context-specific vocalizations. These results have broad implications for research concerning how steroids act at multiple brain loci to regulate distinct socio-sexual behaviors and the associated neuroplasticity.

Significance statement:

Steroid hormones coordinate multiple behaviors into a functional response (reproduction, stress). It is easy to assess such coordination when comparing actions in different target tissues, but is more of a challenge when numerous functions are combined in the brain. Our study illustrates how steroids act on distinct cell groups to regulate separate components of a T-dependent behavior, learned birdsong. T in the medial preoptic nucleus (POM) increased singing but not optimal song performance. Moreover, T in the POM enhanced volumes of forebrain regions that control song, presumably reflecting the effect of activity-dependent plasticity. Thus, optimal performance of a complex, learned behavior requires T at multiple loci and activity and/or transsynaptic influences may stimulate forebrain neuroplasticity.

Introduction

Steroid hormones such as testosterone (T) can have multiple effects on physiology, morphology, and behavior (Adkins-Regan, 2009; Ball et al., 2002; Bottjer and Johnson, 1997; Lee and Pfaff, 2008). These pleiotropic effects of steroids allow coordinating suites of traits into an organized functional response (Arnold, 1981; Pfaff et al., 2008). In the case of the regulation of behavior both motivational and performance aspects of behavior as well cognitive components are often activated by the same hormone (Alexander and Sherwin, 1991; Ball et al., 2002). The neural sites of steroid action coordinating these distinct aspects of an integrated behavioral response have not been well characterized. In this study we investigated the site of hormone action in relation to the activation of different aspects of birdsong to illustrate how such an integrated regulation can occur.

Throughout the songbird brain there are multiple sites of steroid action (Ball et al., 2002; Bernard et al., 1999). For instance, androgen receptors are expressed in HVC, RA, lMAN and throughout the hypothalamus and midbrain and estrogen receptors are expressed in HVC as well as in the hypothalamus. This observation, in line with a plethora of correlational evidence have alluded to the possibility that androgens such as T act in the SCS to activate song in songbirds (Ball et al., 2002; DeVoogd, 1986; Nottebohm et al., 1976; Nottebohm, 1981; Pintér, et al., 2011; Thompson, 2011). However, multiple studies have indicated that the mechanism is not so simple, and steroids may act at multiple levels of the songbird brain to regulate specific aspects of song (Arnold, 1981). For

instance, blocking T actions in HVC reduces song quality but does not affect song rate (Meitzen et al., 2007). Also, implanting T in the HVC or RA of castrated white-crowned sparrows does not activate singing (Brenowitz and Lent, 2002). Moreover, in the classic study by Nottebohm et al (Nottebohm et al., 1976), male canaries with lesioned HVCs produced 'silent song', during which they assumed all the postural components associated with song production but did not produce audible vocalizations. Therefore, while songbirds represent an excellent model system in which to study the distinctive functional roles of steroid hormones, it has not been clearly identified where outside the SCS T acts to regulate the probability of singing or song rate.

The medial preoptic area is an area of the brain that is a key substrate for T to activate male-typical sexual behavior. For instance, studies in rodents and in Japanese quail (*Coturnix japonica*) show that T actions in this brain region are sufficient for activating male-typical sexual motivation and performance (Balthazart and Surlemont, 1990; Johnson and Davidson, 1972). Relevant to the current study, Riters and Ball (1999), using bilateral electrolytic lesions of the medial preoptic nucleus (POM) of male European starlings (*Sturnus vulgaris*), demonstrated that this nucleus is required for increases in song rate that occur in response to the presentation of a female. Hence, the POM is a strong candidate neural substrate for T activating the motivational aspects of song in songbirds.

In the present study we demonstrate that the POM of songbirds is a critical site for the activation of sexual motivation and singing by T, but not for enhancing specific aspects of song stereotypy as well as the attentional components that

coordinate singing with the suite of sexual behaviors. Specifically, we found that T in the POM increases song rate but not the latency and quality aspects (e.g., loudness and stereotypy) of song associated in birds with the systemic action of circulating T. Moreover, T in the POM was sufficient to activate copulation but not calls and peri-copulatory songs in the presence of a female. Finally, T in the POM increased the volumes of SCS nuclei in parallel with the increased singing activity thus providing strong additional evidence of activity-induced brain plasticity in these structures. These results suggest that T acts at multiple levels of the brain to regulate distinct components of complex socio-sexual behaviors and acts at multiple, distinct neural substrates to coordinate the motivational, cognitive, and attentional aspects of sexual behavior.

Results

Serum T concentrations and brain implant location. The two groups of birds that did not receive peripheral T implants had similarly low mean values for serum concentrations of T (See Fig. 1A) and were statistically indistinguishable from one another ($p>0.9$), while birds with peripheral T implants (PER-T) had substantially higher concentrations of circulating T. There was also no difference in plasma T concentration ($p=0.9$) among POM-NO T birds between subjects with empty implants and subjects with T implants outside POM.

The POM-T and POM-NO T groups also possessed POM volumes contralateral to the implant site approximately half the size of PER-T birds (see Methods; POM-T: $2.66 \times 10^5 \pm 1.75 \times 10^4$, POM-NO T: $2.29 \times 10^5 \pm 1.24 \times 10^4$, PER-T: 5.20

x 10^5 ±7.09 x 10^4 μm^3, all Means ± SEM). Volumes in the PER-T group were thus significantly larger than in the other 2 groups ($p<0.001$ in both cases) but these groups were not significantly different ($p>0.56$).

Six out 13 T-filled cannulae contacted the POM (Fig. 1C). T-filled cannulae that missed the POM (n=7; Fig. 1B-D) did not induce singing or other sexual behaviors. The latter group of birds was included in the group called POM-NO T to increase statistical power (n=10; see Methods), reflecting that neither group received T contacting their POM and did not show an activation of song or sexual behaviors. Importantly, among POM-NO T birds there was also no difference in contralateral POM volume between birds implanted with an empty or a T-filled cannula (2.02 x 10^5 ±5.26 x 10^3 μm^3 and 2.46 x 10^5 ±1.29 x 10^4 μm^3, respectively; $p>0.17$).

T in the POM increases song rate but not song quality. PER-T and POM-T birds produced long, complex songs compared to POM-NO T birds (Fig. 2A). Repeated measures ANOVAs revealed a significant main effect of treatment on song rate measures (song rate, % time singing, and song duration; $p<0.001$) and a significant interaction of day and treatment for these measures ($p<0.05$). The results of post-hoc analyses are in Figure 2. PER-T birds began singing at high rate before the other groups and their songs had a longer song duration (Fig. 2B-D). Eventually, POM-T birds began singing with indistinguishable song rate, % time singing, and song duration compared to PER-T birds, and both groups had larger values for these variables compared to POM-NO T in the latter half of the observations. Linear contrasts revealed that POM-T birds showed a linear

increase in all of these measures ($p<0.05$), while this was not exhibited by the other groups.

On average during days 5 to 11, PER-T birds sang songs that were more than twice as loud (energy, post-hoc Tukey's, $p=0.043$) and of higher quality (bandwidth CV and entropy variance CV, post-hoc Tukey's $p<0.05$) compared to POM-T birds (See Methods; Fig. 2 E-G). These results as a whole suggest that T in the POM is sufficient to increase song rate, but globally circulating T increases song rate more rapidly (i.e. within 1 to 3 days) and enhances the full suite of acoustic and quality measures characteristic of male canary song.

T in the POM induces SCS growth and singing rate predicts HVC and RA volume. After observing the substantial induction of song rate in POM-T birds, we wondered whether the song control system had changed in response to this treatment. POM-NO T birds had smaller song control nuclei than both other groups (HVC, RA, and Area X; post-hoc Tukey's $p<0.05$) while PER-T and POM-T were indistinguishable from each other (Fig. 3A). These results suggested that T action in the POM leads to song control nuclei growth indirectly via increased singing activity.

Accordingly, a forward regression analysis identified song rate as a significant predictor of HVC and RA volume (see Methods; $r=0.533$ and 0.477 respectively; $p<0.05$; Fig. 3B-C). These results are fully consistent with the notion that the substantial increase in song control system volume in POM-T birds may be in part due to singing activity.

T in the POM induces copulation but not vocalizations in the presence of a female. There was a main effect of treatment on the copulation rate (Kruskal-Wallis ANOVA, p=0.02). Specifically, POM-T birds attempted to copulate more frequently compared to POM-NO T birds (post-hoc Mann Whitney, p=0.038; Fig. 4A). Moreover, PER-T birds copulated more frequently than POM-NO T birds (post-hoc Mann Whitney, p=0.007) and were not different from POM-T birds (post-hoc Mann Whitney, p>0.05). There was also a main effect of treatment on call rate (p=0.002) and peri-copulatory songs (p=0.05, See Methods for definition; Kruskal-Wallis ANOVA). Interestingly, POM-T birds were not different from POM-NO T birds for these two measures (call rate, p=0.92; peri-copulatory song, p=0.20; post-hoc Mann Whitney, Fig. 4B-C), whereas PER-T birds were significantly different from POM-NO T for both measures (call rate, p=0.001; peri-copulatory song, p=0.02; post-hoc Mann Whitney). PER-T birds also had a higher call rate compared to POM-T birds (p=0.006) but were not different from POM-T in terms of peri-copulatory song (post-hoc Mann Whitney, p=0.19). This suggests T acting in other areas of the brain is required to orchestrate vocalizations specific to the sexual context.

Discussion

Steroid hormones can induce variation in physiological state that dramatically affects responses to socially relevant stimuli (Adkins-Regan 2009; Harding, 2004; Maney and Pinaud 2011). Steroids can also modify cognitive processes ranging from attention (McCall and Singer, 2012) to learning (Ball et al., 2002; Bottjer and Johnson, 1997) that affect the probability and quality of a particular

behavioral response. Our studies of the neural basis of how T regulates song behavior illustrate two important principles. The first is that T, like other steroid hormones, can regulate different aspects of the same behavior or the expression of a given behavior in different contexts by acting independently in different brain areas. The second is that T-induced changes in behavioral activity can feed back on the morphology and physiology of brain areas involved in the production or regulation of sensorimotor aspects of the behavior of interest. Studies of steroid action on courtship song are especially amenable to this sort of analysis.

One implication of the pioneering study by Nottebohm et al (1976) not widely appreciated at the time is that the SCS regulates the learning and production of song while areas outside of this system regulate the probability that song will occur. Canaries with HVC lesions tried to sing but could not produce the vocal output (what Nottebohm referred to as “silent song”; 1976). At that time, the discovery of steroid receptors in several song control nuclei (Arnold et al., 1976; Ball et al., 2002; Lieberburg and Nottebohm, 1979; Metzdorf et al., 1999) drew attention to the action of steroids directly in the SCS (Arnold et al., 1976; Metzdorf et al., 1999) and it is only later that research suggested that T is acting potentially at multiple sites to regulate distinct components of song behavior (Arnold, 1981; Ball et al., 2002; Brenowitz and Lent, 2002; Meitzen et al., 2007). In this study, we conclusively identify for the first time a neural substrate outside of the SCS where T acts to modulate song. Specifically, T in POM was sufficient to enhance the rate of song production but did not lead to the production of fully stereotyped vocalizations typical of birds systemically exposed to T. Thanks to

the availability of extensive video recording, we also confirm for the first time in songbirds the role of T action in the preoptic area on male-typical copulatory behavior, previously described in other avian and in mammalian species (Paredes, 2003; Riters, Absil, and Balthazart, 1998; Watson and Adkins-Regan, 1989).

Dissociations similar to these observed here in the control of singing have been observed in castrated rodents implanted with T solely in their medial preoptic area, resulting in an enhancement in copulation but a lack of the vigor and performance present in intact animals (Wood and Newman, 1995). This study goes a step further, by demonstrating the dissociability between the motivation to perform a complex social behavior and its quality as well as its coordination with relevant stimuli (the female). It is likely that T acting at neural substrates in multiple areas of the social behavior network (Kingsbury, et al., 2011; Newman, 1999) that includes the POM, as well as in the SCS itself (Ball et al., 2002) is required for the occurrence of the full suite of reproductive behaviors present in songbirds.

This study also provides credence to the hypothesis that singing in and of itself has stimulating effects on neuroplasticity (Alvarez-Borda and Nottebohm, 2002; Sartor and Ball, 2005). Recently it has been debated if the effects of singing activity can be separated from the effects of circulating T on changes in SCS morphology (Elizabeth Adkins-Regan, 2005; Sartor and Ball, 2005). Here we show that with substantial increases in song rate in the absence of globally circulating T and in the absence of T in the brain outside of its implantation in the

POM, the SCS undergoes robust changes in size, supporting the notion of activity-induced neural growth and plasticity. For instance, the volume of the contralateral POM, which is positively associated with exposure to T (Charlier et al., 2008; Riters et al., 2000), was about twice as large in birds exposed to global T compared to castrated birds with T solely in their POM. Hence, our study provides strong evidence that singing activity can lead to robust neuroplasticity in the SCS independently of T.

These results also corroborate models of T actions indicating that T works both directly and indirectly to change the SCS and song activation (Ball et al., 2002). Moreover, these data raise the question of how T in the POM exerts these effects: transsynaptic influences are possible, as indirect connections exist between the POM and HVC and RA, via the periaqueductal grey and the ventral tegmental area (Appeltants, et al., 2000; Appeltants, Ball, and Balthazart, 2002; Riters and Alger, 2004). It is however more likely that the observed plasticity is a direct consequence of the singing activity itself (Alvarez-Borda and Nottebohm, 2002; Li et al., 2000). Future investigations on these respective and possibly interacting hypotheses are thus of the utmost importance.

A basic principle of steroid hormone action that was identified early in the history of the field is that multiple functions must be coordinated to organize a functional response such as reproduction or stress (Beach, 1948). It is easy to assess such coordination when comparing actions in very different target tissues such as the brain vs. the periphery. However, when multiple functions are combined to a single target tissue such as the brain it is more of a challenge. Despite the fact

that steroids have been directly implanted into the brain since the 1960s there are still few examples that illustrate specialization of steroid action in multiple brain sites for the coordination of a single behavior (Pfaff et al., 2008). Our study illustrates how steroids can act on distinct cell groups to regulate separate components of a single behavioral response. T acting in the POA clearly can motivate song but it cannot insure that song performance is optimal. However, just increasing song activity via action in the POA results in enhanced volumes of the key song nucleus HVC. This type of coordination can be important in regulating many social behaviors, especially those depending on experience.

Materials and Methods

Animals and pre-experimental manipulations. Canaries (*Serinus canaria*) of the Border strain were used for this study. Male and female canaries were obtained from a local breeder (Maryland Exotic Birds). Upon entry into the lab birds were placed on a short day (SD) photoperiod (8L:16D) for six weeks to induce photosensitivity (Dawson et al., 2001). Birds in a photosensitive but not photostimulated state also possess regressed testes, which facilitates effective castration.

Male birds were castrated by deeply anesthetizing them with isoflurane gas (IsoSol isoflurane, Vedco, Inc., St Joseph, MO, USA; Isotec 4 anesthesia machine, Surgivet, Inc., Waukesha, WI, USA) and placed on their right side. The left testis was then removed through an incision below the last rib; immediately after, the bird was placed on its left side and the right testis was removed in an

identical manner. After recovery from surgery, birds were placed under a heat lamp until they perched. Then, birds were placed back in their home cages and allowed to recover for six weeks, to allow adequate time for the physiological and behavioral effects of T to disappear.

Experimental groups and stereotaxic implantation. Birds were anesthetized using isoflurane gas and placed in a stereotaxic apparatus modified for use in small birds such as canaries with the beak holder placed 45° below the horizontal axis of the apparatus. We used the following stereotaxic coordinates to target the POM: dorso-ventral: -7 mm from the dorsal surface of the brain; anterior-posterior: 2.3 mm from the rostral tip of the cerebellum; and medial-lateral: ± 0.15 mm from midline. Each bird received a unilateral implant aimed at the POM using a Hamilton syringe fashioned to hold the 27-gauge cannula filled with T or left empty. Cannulae were lowered to the target coordinates and dental cement was applied around the implant. Excess portion of the cannula was clipped off after the cement had dried. The skin was then sutured over the implant and lidocaine and antibiotics were applied around the sutured portion of the skin using a Q-tip.

Implants were made using blunted 27-gauge needles filled over a length of 1 mm with crystalline T (Sigma T 1500; Carere et al., 2006). Implants were cleaned using acetone and a Kimwipe to remove any hormone that stuck to the outside of the tube. The side of the brain in which the implant was made was randomized across birds. Once birds recovered, they were returned to individual, sound-attenuated chambers set to 14L:10D to simulate breeding photoperiods.

Due to expected variation in implant sites (Balthazart and Surlemont, 1990), we implanted 13 birds with a T-filled cannula and 3 with an empty cannula. Pilot studies indicated when T missed the POM, no song or copulatory behavior was induced and accuracy of the implants was about 50%. Therefore, when T-filled cannulae missed the POM, we lumped the birds with T-filled cannulae that missed the POM and birds that received empty cannulae into the same group, called POM-NO T, reflecting that neither group received T contacting the POM. We confirmed by t tests that these groups did not differ significantly on any morphological (see Methods; SCS nuclei volumes, $p \geq 0.40$; contralateral POM volumes, $p > 0.17$), physiological (T concentrations, $p \geq 0.90$) or behavioral measure (all $p \geq 0.84$). In the end, 6 out of 13 T-filled cannulae contacted the POM.

Overall, this experiment had three groups: group 1 was administered T peripherally, by implanting each subject subcutaneously with an 8-mm long Silastic™ implant (Dow Corning; internal diameter=0.76 mm, external diameter=1.65 mm) filled with 6-mm of crystalline T (PER-T; n=4); group 2 received a cannula filled with T that contacted the POM (POM-T; n=6); group 3 received a cannula that was either empty (n=3) or filled with T but missed the POM (n=7) (POM-NO T; n=10, see above). All birds received either a T-filled or empty brain cannula and a T-filled or empty Silastic™ implants; the contents of cannulae or implants varied based on the treatment group.

Song and other sexual behaviors. Each day video and audio recordings were made from 800h to 930h (lights on at 800 h), 1300h to 1430h, and 1600-1730h.

The following song behaviors were quantified from recordings made every other day during 11 days, starting after 3 days of treatment: song rate (songs/hour), mean duration of each song, % of time spent singing, entropy variance, energy, fundamental frequency, and bandwidth (Derégnaucourt, et al., 2005; Tchernichovski et al., 2000). Songs were defined as vocalizations being longer than or equal to 1 second in duration and separated by 500 milliseconds of silence (Alward et al., 2014; Voigt and Leitner, 2008).

We also quantified song stereotypy. Song stereotypy indicates how similar certain features of song are across song renditions. Song stereotypy was determined by calculating the coefficient of variation (CV=(SD/AVG)*100)) using the standard deviations of song acoustic features (SD) described above and dividing this by the average (AVG) across the same values used to calculate the SD. CV is an inverse measure of song stereotypy (Meitzen et al., 2007; Meitzen, et al., 2009).

Fourteen days after the beginning of treatment (3 days post-treatment plus the 11 days of song recording while males were alone), each male canary was presented with a female that had been implanted with a 14-mm Silastic™ implant filled with 12 mm of crystalline 17β-Estradiol (Sigma). Notably, when male canaries are presented with a female, song production ceases (Alward et al., 2014). They were housed with the female for three days during which time their behavior was recorded in the same way as described for the song behaviors. The following behaviors were quantified: proximity initiations (i.e. approaching the female to less than one body length), bill touches, copulation attempts, songs,

calls, and peri-copulatory songs. Calls are much shorter and simpler vocalizations compared to song (Marler and Slabbekoorn, 2004). Peri-copulatory songs are songs that are produced immediately before and during copulation in male canaries. We also quantified non-social behaviors such as feeding, drinking, and grooming.

Brain collection and verification of implants and castrations. Sixteen days after treatment initiation, birds were deeply anesthetized (4% Isoflurane), weighed, and their brain was extracted and fixed in acrolein after collecting blood from the trunk region into 1.5 ml centrifuge tubes. Blood was spun down at 8,000 rpm for 6 minutes and serum was collected and placed at -20° C. Brains were agitated in acrolein for 2 hours, then washed for 15 minutes four times in phosphate buffered saline (PBS) and placed in sucrose (30% solution in PBS) over night until they sank to the bottom of the vial. After cryoprotection by sucrose, brains were flash frozen in dry ice for 5 minutes, and then placed into a -70° C freezer. At autopsy, all birds were found to be completely castrated and no testicular remnants or regrowth could be detected.

Brain and serum analyses. Brains were sectioned using a cryostat at 30 microns into four series of sections that were stored in cryoprotectant. These four series were placed into a -20° C freezer. One series was later mounted on gelatin-coated slides and exposed to air for a day. Then, mounted sections were exposed to a standard Nissl staining procedure and coverslipped using Permount (Fisher Scientific). Based on these stained sections, the positions of the implant

centers were drawn onto a series of modified atlas plates obtained from the canary atlas made by Stokes et al (Stokes, Leonard, and Nottebohm, 1974).

Concentrations of serum T were determined using an enzyme-linked immunosorbent assay ELISA (Enzo Life Sciences, Testosterone ELISA kit, catalog #ADI-900-065, Plymouth, PA). This allowed us to determine if there was any detectable leakage of T from the brain cannula into the peripheral circulation as well as the efficacy of the Silastic™ implants and castrations.

SCS and POM volume reconstruction. As stated above, T in the POM was found to induce song rate to levels of PER-T birds. We took advantage of this observation to assess whether high song rate in the absence of global T action could induce increases in the volume of song control system nuclei. To this aim, photomicrographs of Area X, HVC, and RA were taken at 2.5x magnification in the Nissl-stained sections. The area of each nucleus was determined in both hemispheres of each section where it appeared using NIH Image J and volumes were determined by multiplying areas by the section thickness, summing these values, and then multiplying this value by 4, since only every 4th section was Nissl stained (Sartor et al., 2005). In line with previous work on canaries, no systematic assymetries were found between the hemispheres (F Nottebohm and Arnold, 1976; Sartor et al., 2005). This would not be expected as the song nuclei in both hemispheres are active during song production (McCasland, 1987). One bird in the POM-NO T group was an outlier for HVC and RA volumes (>2.5 standard deviations from the mean) and was thus removed from the ANOVA analysis and from the regression of singing activity on RA volumes (its RA

volume was extraordinarily high and unexplainable based on our treatment groups and all other measures; it should be noted that multiple factors, including age, can influence SCS volume size; see Introduction and ref. Bernard, Eens, and Ball, 1996). One PER-T bird had damaged sections that precluded analysis of RA and was therefore excluded from analysis of this brain region. We also quantified the volume of the POM in one section at the level of the anterior commissure contralateral to the implant sites using the same method as above in each bird. The volume of the POM is highly sensitive to the concentrations of T (Charlier et al., 2008; Riters et al., 2000), with T correlating positively with POM volume. Thus, the volume of the POM is a critically sensitive marker of T present in the cerebrospinal fluid (CSF) and general circulation, and was thus used as a proxy for the efficacy and specificity of our central and peripheral T implants.

Statistical Analyses. ANOVAs (Kruskal-Wallis if homogeneity of variance was not met) were used to assess the effects of treatment and/or day on all measures. A two-way (day*treatment) ANOVA was used to assess how treatment affected song rate, % time spent singing, and duration over time. ANOVAs were conducted on individual days when an interaction was observed. Five or six out of 6 POM-T birds consistently sang from day 5 to 11; before this a very small number of birds sang and a maximum of 2/10 birds in the POM-NO T group sang throughout the whole experiment. To avoid arbitrary value assignments to acoustic/stereotypic measures and removing birds from these analyses, we compared the PER-T and POM-T birds in terms of these features collapsed over days 5-11. A t-test was used to make these comparisons. Tukey's

or Mann Whitney tests were used for post-hoc pairwise comparisons following significant omnibus parametric and non-parametric ANOVAs, respectively. A linear regression was also used to test whether singing activity predicted the volumes of the SCS nuclei. Specifically, song rate and % time spent singing were entered into a model as predictors for the volumes of HVC, RA, and Area X. Stepwise regression was used to determine which, if any, of these two variables are significant predictors of SCS nuclei volume.

Acknowledgements: This work was supported by an NIH/NINDS RO1 35467 to GFB and grant SSTC PAI P7/17 from the Belgian Science Policy (BELSPO) to JB and GFB. We would like to thank Dr. Farrah Madison for running the T assay, and Adam Podlisky, Kathryn Rownd, Hayley Weidenbenner, and Trevor Chan for technical assistance.

Figure legends

Figure 1. Concentrations of T and T implant sites. A) Concentrations of T in the 3 treatment groups, PER-T, POM-T, and POM-NO T. *= $p<0.05$ vs. POM-NO T; #= $p<0.05$ vs. POM-T. B-E) Implant sites in intermediate POM (iPOM; B), caudal POM (cPOM; C), near ventromedial nucleus of the hypothalamus (VMN; D) and in dorsal thalamus near tractus occipitomesencephalicus (OM) and nucleus spiriformis medialis (SPM; E). White dashed lines demarcate the POM. White circle filled in black indicate T implants that did not contact the POM; white ones indicate implants that contacted the POM; diamonds indicate empty implants DS, supraoptic decussation; AC, anterior commissure.

Figure 2. Effect of treatments on song rate, duration, and quality. A) Representative spectrograms of song from each group. B-G) Effects of T treatment on various song measures. B-D show the effects of treatment on measures of song rate and duration; E-G represent average quality features of song. *= $p<0.05$ vs. POM-NO T; #= $p<0.05$ vs. POM-T.

Figure 3. Effects of treatments on SCS volume (A) and relationship between song rate and HVC and RA volume (B-C). Trend lines indicate a significant regression. Different symbols were used to indicate data from the 3 experimental groups. In panel A: *= $p<0.05$ vs. POM-NO T.

Figure 4. Effects of treatment on behaviors in the presence of a female. *= $p<0.05$ vs. POM-NO T; #= $p<0.05$ vs. POM-T.

Figure 1

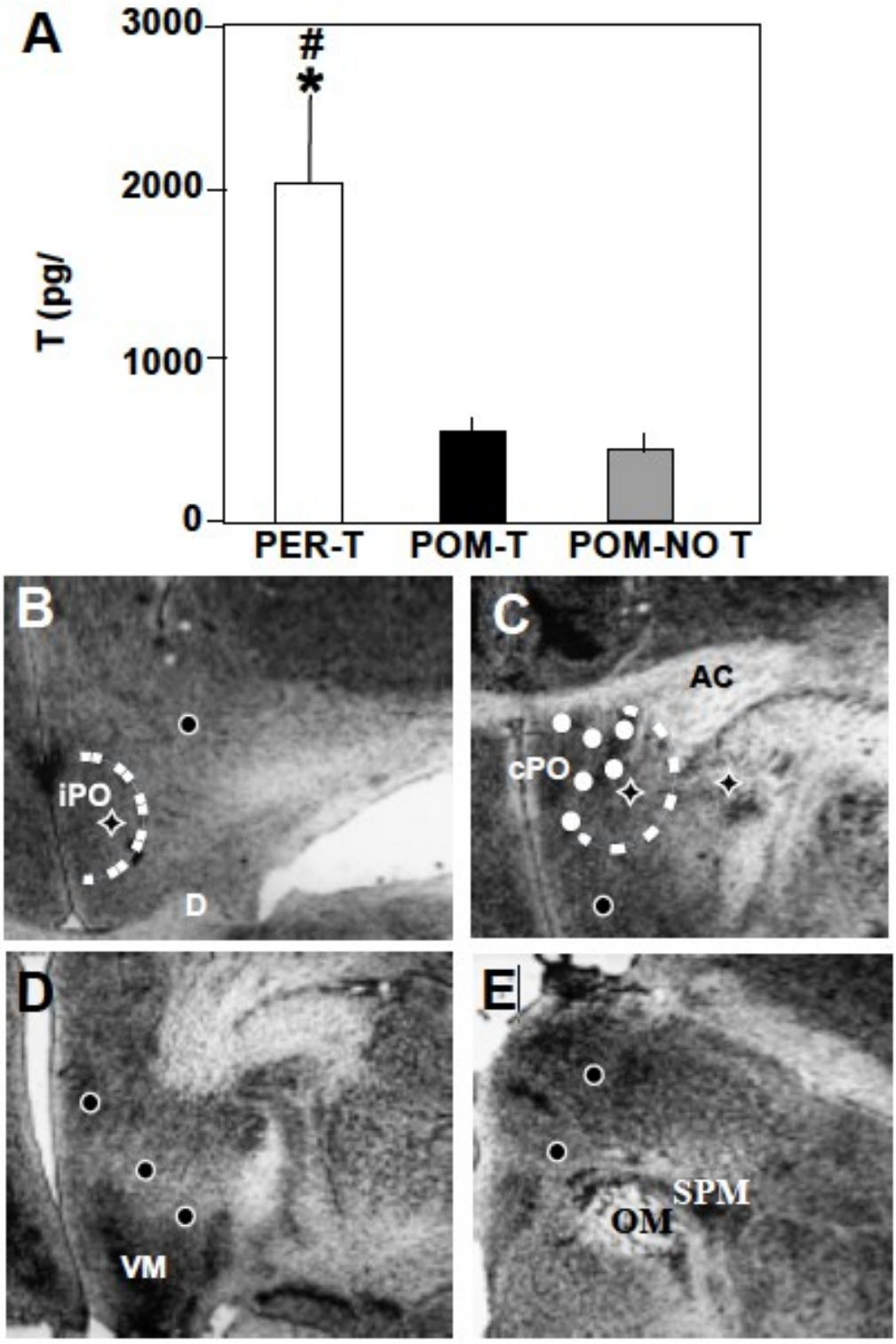

A
3000
2000
1000
0
T (pg/
#
*
PER-T
POM-T
POM-NO T
B
iPO
D
C
AC
cPO
D
VM
E
SPM
OM

Figure 2

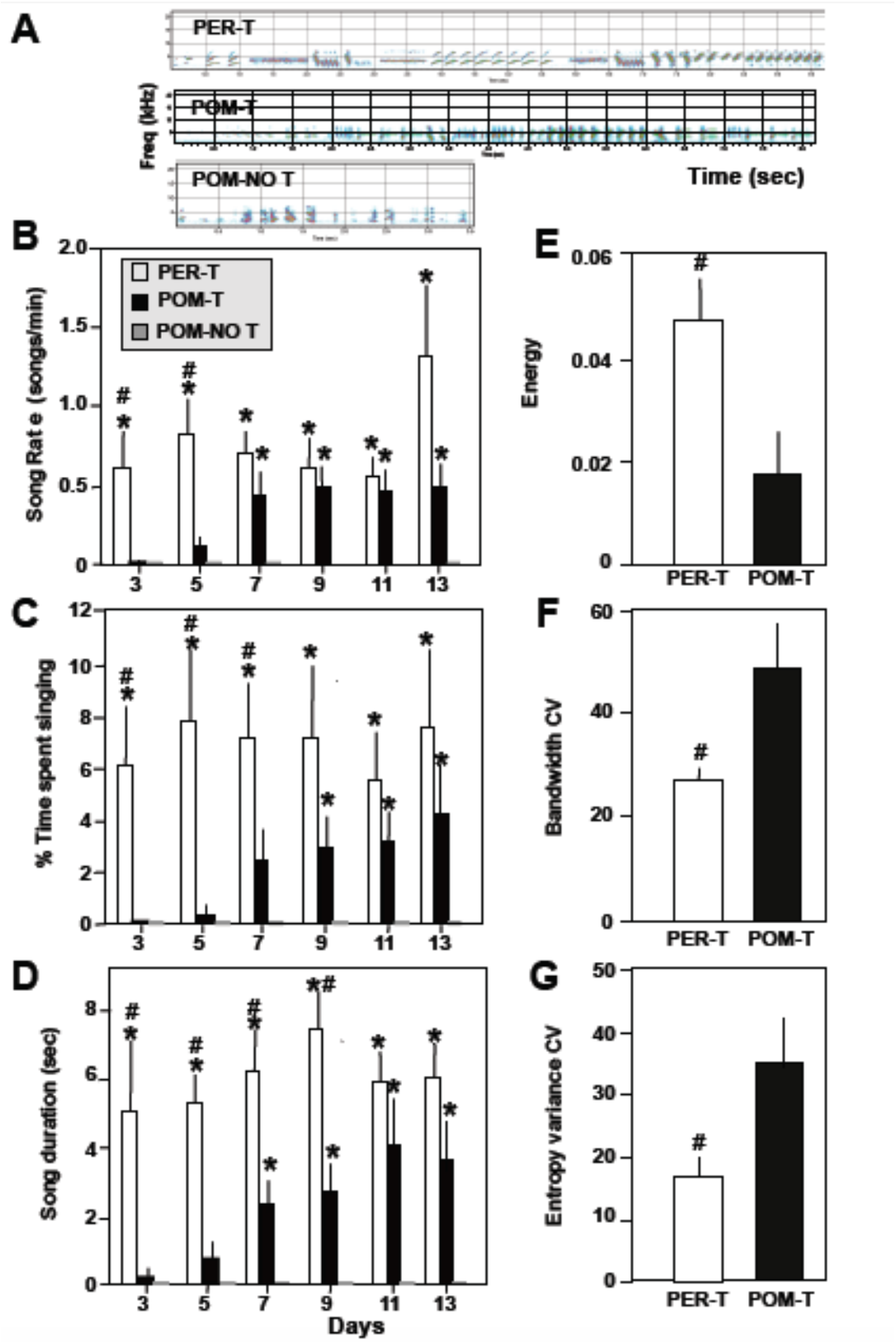

A
PER-T
POM-T
POM-NO T
Freq (kHz)
Time (sec)
B
PER-T
POM-T
POM-NO T
Song Rat e (songs/min)
C
% Time spent singing
D
Song duration (sec)
Days
E
Energy
F
Bandwidth CV
G
Entropy variance CV

Figure 3

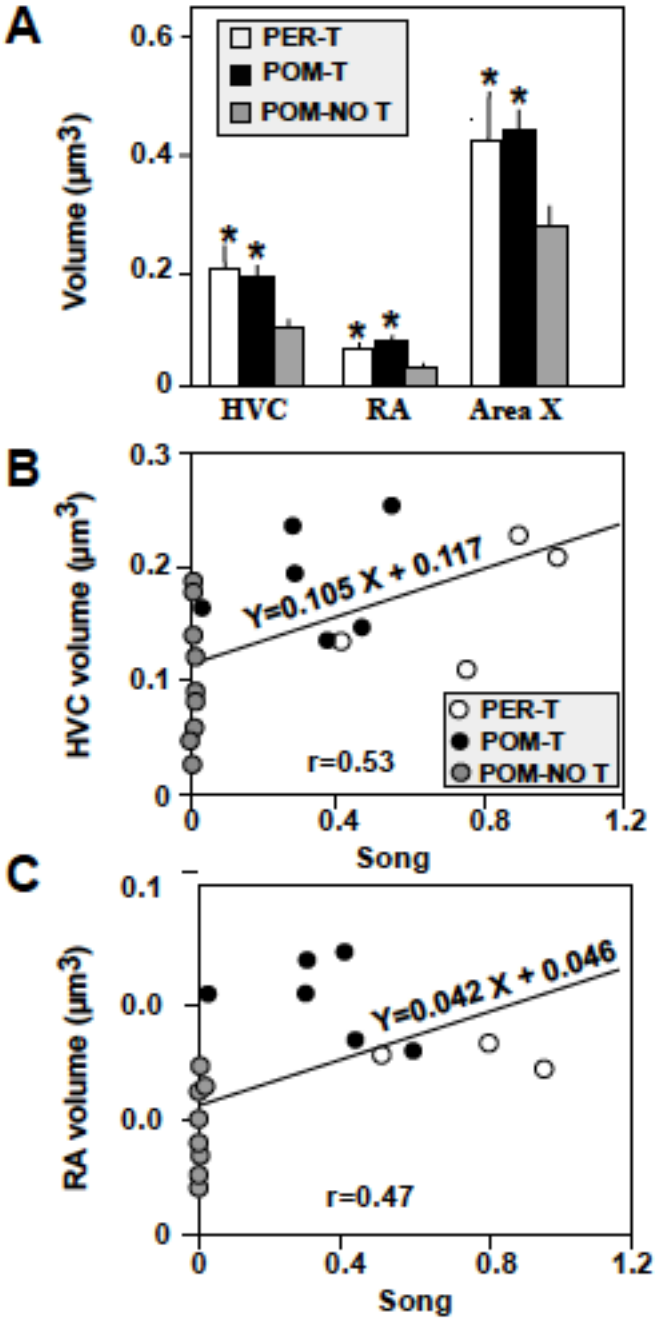
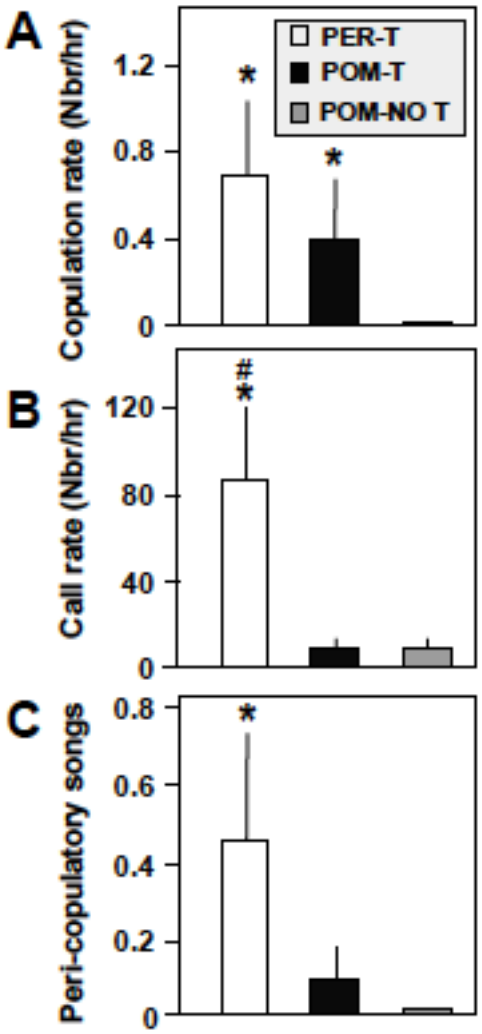

A
PER-T
POM-T
POM-NO T
Volume (µm3)
0.6
0.4
0.2
0
HVC
RA
Area X
B
HVC volume (µm3)
Y=0.105 X + 0.117
r=0.53
PER-T
POM-T
POM-NO T
0.3
0.2
0.1
0
0
0.4
0.8
1.2
Song
C
RA volume (µm3)
Y=0.042 X + 0.046
r=0.47
0.1
0.0
0.0
0
0
0.4
0.8
1.2
Song

Figure 4


A
Copulation rate (Nbr/hr)
1.2
0.8
0.4
0
PER-T
POM-T
POM-NO T
*
*
B
Call rate (Nbr/hr)
120
80
40
0
*#
C
Peri-copulatory songs
0.8
0.6
0.4
0.2
0
*

Chapter 5: The role of testosterone action in HVC in the regulation of singing and neuroplasticity

Introduction

The previous experiment has demonstrated that the actions of T in various brain nuclei and in the periphery in the regulation of a complex behavior such as birdsong, as well as its underlying neural circuitry, are non-redundant. Specifically, T in the POM is sufficient for activating the motivational measures associated with song but did not activate the amplitude and stereotypy of song. Moreover, singing in and of itself was able to enhance the volumes of key nuclei in the SCS.

Based on previous work by Meitzen and colleagues (Meitzen et al., 2007), it is plausible that T acting in HVC is required for an enhancement of song stereotypy. For instance, blocking androgen receptors in HVC reduces song quality but does not affect song rate (Meitzen et al., 2007). In the present study, we investigated the role of T in the HVC in the regulation of these acoustic features of song in canaries. To this end, castrated canaries were implanted with T simultaneously in their POM and HVC and we assessed the stereotypy and complexity of songs, both features of songs that are thought to be regulated by T acting outside of areas such as the POM that regulate the motivational measures of singing (Chapters 3 and 4). We also implanted a group of birds with T only in HVC; based on the results from these treatment groups we will be able to assess the independent and possibly interactive roles of T and singing in the regulation

of SCS neuroplasticity (as assessed by both Nissl-defined volumes and new neuron incorporation in HVC).

Methods

Materials and Methods

Animals and pre-experimental manipulations. Canaries (*Serinus canaria*) of the Border strain were used for this study. Male and female canaries were obtained from a local breeder (Maryland Exotic Birds). Upon entry into the lab birds were placed on a short day (SD) photoperiod (8L:16D) for six weeks to induce photosensitivity (Dawson et al., 2001; Nicholls et al., 1977). Birds in a photosensitive but not photostimulated state also possess regressed testes, which facilitates effective castration. All procedures were approved by the Johns Hopkins University Animal Care and Use Committee.

Male birds were castrated by deeply anesthetizing them with isoflurane gas (IsoSol isoflurane, Vedco, Inc., St Joseph, MO, USA; Isotec 4 anesthesia machine, Surgivet, Inc., Waukesha, WI, USA) and placed on their right side. The left testis was then removed through an incision below the last rib; immediately after, the bird was placed on its left side and the right testis was removed in an identical manner. After recovery from surgery, birds were placed under a heat lamp until they perched. Then, birds were placed back in their home cages and allowed to recover for six weeks, to allow adequate time for the physiological and behavioral effects of T to disappear.

Experimental groups and stereotaxic implantation. Birds were anesthetized using isoflurane gas and implanted with a silastic implant as in chapters 3 and 4. Birds were then placed in a stereotaxic apparatus modified for use in small birds such as canaries with the beak holder placed 45° below the horizontal axis of the apparatus. We used the following stereotaxic coordinates to target the POM: dorso-ventral: -7 mm from the dorsal surface of the brain; anterior-posterior: 2.3 mm from the rostral tip of the cerebellum; and medial-lateral: ± 0.15 mm from midline. For the HVC, the following coordinates were used: dorso-ventral: -0.60 mm from the dorsal surface of the brain; anterior-posterior: -0.20 mm from the rostral tip of the cerebellum; and medial-lateral: ± 2.70 mm from midline. Each bird received ipsilateral, unilateral implants aimed at the POM and HVC using a Hamilton syringe fashioned to hold the 27-gauge cannula filled with T or left empty. Cannula were lowered to the target coordinates and dental cement was applied around the implant. Excess portion of the cannula was clipped off after the cement had dried. The skin was then sutured over the implant and lidocaine and antibiotics were applied around the sutured portion of the skin using a Q-tip.

Implants were made using blunted 27-gauge needles filled over a length of 1 mm with crystalline T (Alward et al., 2013; Balthazart and Surlemont 1990; Sigma T 1500). Implants were cleaned using acetone and a Kimwipe to remove any hormone that stuck to the outside of the tube. The side of the brain in which the implant was made was randomized across birds. Once birds recovered, they were returned to individual, sound-attenuated chambers set to 14L:10D to simulate breeding photoperiods.

Due to expected variation in implant sites (Alward et al., 2013; Balthazart and Surlemont, 1990), we implanted 15 birds with T-filled cannula targeting both the HVC and POM, 7 birds with T contacting their POM only, 2 birds with T contacting only their HVC, and 2 birds with empty cannula targeting both HVC and POM. Our previous work has shown that when T missed the POM, no song or copulatory behavior was induced and accuracy of the implants was about 60% (Alward et al., 2013). Therefore, when T-filled cannulae missed the POM, we lumped the birds with T-filled cannulae that missed the POM and birds that received empty cannulae into the same group, called POM-NO T, reflecting that neither group received T contacting the POM. We proceeded similarly when T missed the HVC (Meitzen et al., 2007). We confirmed by t tests that these groups did not differ significantly on any morphological (see Methods; SCS nuclei volumes, contralateral POM volumes), behavioral, or physiological level.

Overall, this experiment had five groups: 8 birds received T implants contacting the HVC and POM (one had testicular remnants and was excluded from the analysis; see below) (HVC-POM T, n=7); 8 birds received T contacting their POM but not their HVC (POM-T, n=8); 5 birds had T contacting their HVC and not their POM (HVC-T, n=5); and 4 birds did not have T contacting their HVC or POM (HVC-POM NO T, n=4). HVC-T and HVC-POM NO T did not sing except for 1 or 2 birds singing very infrequently and were thus not included in the song acoustic analysis. Eight birds received subcutaneous T implants, but one of them had testicular remnants and were removed from the analysis (see below) (PER-T, n=7). All birds received two brain cannulae and a T-filled or empty Silastic™

implants; the contents of cannulae or implants varied based on the treatment group.

Song features. Song recording and analysis settings (e.g., filter settings, sampling rate, etc) were identical to those in chapter 2. Each day video and audio recordings were made from 800h to 930h (lights on at 800 h), 1300h to 1430h, and 1600-1730h. We quantified the song features as listed in the Appendix collapsed across the three final days of treatment using the sound analysis program Avisoft (Alward et al., 2013). Songs were defined as vocalizations being longer than or equal to 1 second in duration and separated by 500 milliseconds of silence (Alward et al., 2013; Voigt and Leitner, 2008).

We also quantified song stereotypy as above (Chapters 3 and 4; Meitzen et al., 2007).

Brain collection and verification of implants and castrations. Fourteen days after treatment initiation, birds were deeply anesthetized (4% Isoflurane), weighed, and their brain was extracted and fixed in acrolein after collecting blood from the trunk region into 1.5 ml centrifuge tubes. Blood was spun down at 8,000 rpm for 6 minutes and serum was collected and placed at -20° C. Brains were agitated in acrolein for 2 hours, then washed for 15 minutes four times in phosphate buffered saline (PBS) and placed in sucrose (30% solution in PBS) over night until they sank to the bottom of the vial. After cryoprotection by sucrose, brains were flash frozen in dry ice for 5 minutes, and then placed into a -70° C freezer. At autopsy, any birds that were incompletely castrated or showed

testicular regrowth were removed from the experiment. One bird treated with HVC-POM T and one bird treated with PER-T showed testicular remnants and were thus excluded.

Brain and serum analyses. Brains were sectioned using a cryostat at 30 microns into four series of sections that were stored in cryoprotectant. These four series were placed into a -20° C freezer. One series was later mounted on gelatin-coated slides and exposed to air for a day. Then, mounted sections were exposed to a standard Nissl staining procedure and coverslipped using Permount (Fisher Scientific). Based on these stained sections, the positions of the implant centers were drawn onto a series of modified atlas plates obtained from the canary atlas made by Stokes et al (1976) using the revised nomenclature for the songbird brain (Reiner et al., 2004). Another series was used for doublecortin (DCX) immunohistochemistry (see below).

Concentrations of serum T were determined using an enzyme-linked immunosorbent assay ELISA (Madison et al., 2015; Enzo Life Sciences, Testosterone ELISA kit, catalog #ADI-900-065, Plymouth, PA). This allowed us to determine if there was any detectable leakage of T from the brain cannula into the peripheral circulation as well as the efficacy of the Silastic™ implants and castrations.

Doublecortin immunohistochemistry and quantification. We used a DCX immunocytochemistry protocol previously used in canary brains (Yamamura et al., 2011). Sections were washed in 0.01M PBS three times, once in 0.1%

sodium borohydride in 0.01M PBS, and 3 times in 0.01M PBS with 1% Triton X (PBST). Endogenous peroxidases were blocked using 0.6% H_2O_2 in PBST for 20 minutes which was followed by three washes in PBST and additional blocking using 10% normal horse serum (NHS) in PBST for 30 minutes. Sections were incubated at 4°C in 2% NHS and PBST and primary antibody (1:5,000 Horse-anti goat, Doublecortin, Santa Cruz, cat#; sc-8066). Sections were washed three times in PBST, then incubated in avidin biotin horseradish-peroxidase complex (Vectastain ABC Elite Kit, 1:200) for 1 hour, and washed three times in PBST. The peroxidase was then visualized using diamniobenzidine (Sigma Fast DAB) for 5 minutes and sections were subsequently washed in 0.01M PBS and mounted onto gelatin-coated microscope slides. Slides were serially dehydrated in ethanol and placed in xylene for 10 min before being coverslipped using Permount (Fisher, Fair Lawn, NJ).

We counted the two different types of DCX-ir cells (Figure 3A)--round and fusiform--in HVC. The round cells are new neurons that have migrated to their final site and begun differentiating while the fusiform cells are still migrating and have not begun differentiating. The counting method was similar to that used by others (Alward et al., 2014; Balthazart et al., 2008; Yamamura et al., 2011). Specifically, DCX-ir cells were counted at three different rostro-caudal levels in three separate fields positioned in the center of HVC and in the adjacent nidopallium lateral and ventral to HVC (see Figure 1 in Balthazart et al*.,* 2008). These three rostro-caudal levels of HVC were roughly equally spaced in the nucleus to provide an overall representation of DCX-ir cells in this structure

(Balthazart et al*.,* 2008*)* and they show significant levels of neuronal incorporation in adulthood (Balthazart et al., 2008; Boseret et al., 2007; Kirn et al., 1999; Yamamura et al., 2011). Immunoreactive cells were manually counted on images digitized through the microscope (20 X objective) in a standardized square area (200 X 200 µm) in each brain region of interest. The Cell Counter function of ImageJ software (version 1.40g; Wayne Rasband, National Institutes of Health) was used to identify DCX-ir cells, which were then classified as round or fusiform by a human observer. The area used for quantification was positioned within the structure of interest in a standard manner using clearly defined brain landmarks as previously described in detail (Balthazart et al., 2008; Yamamura et al., 2011). All immunoreactive cells that contained a clear unstained nucleus surrounded by stained cytoplasm were counted manually. Cells were counted in both hemispheres to allow for interhemispheric comparisons. We also counted the two cell types in comparable brain regions, lateral and ventral to HVC, to confirm specificity of changes in HVC. Cell counts in each area (HVC, lateral or ventral to this nucleus) were added across the three rostro-caudal level (i.e., in three 200 X 200 µm fields or 3 x 0.04= 0.12 mm^2) and expressed as numbers of cells per mm^2.

SCS and POM volume reconstruction. Photomicrographs of Area X, HVC, and RA were taken at 2.5x magnification in the Nissl-stained sections. The area of each nucleus was determined in both hemispheres of each section where it appeared using NIH Image J and volumes were determined by multiplying areas by the section thickness, summing these values, and then multiplying this value

by 4, since only every 4th section was Nissl stained (Alward et al., 2013; Sartor et al., 2005). We also quantified the volume of the POM as it extends from its intermediate position (the DSV extends across the basal level of the brain after the TSM split disappears) to its caudal level (the anterior commissure extends across the midline fully). The volume of the POM is highly sensitive to the concentrations of T (Alward et al., 2013; Charlier et al., 2008; Riters et al., 2000), with T correlating positively with POM volume. Thus, the volume of the POM is a critically sensitive marker of T present in the cerebrospinal fluid (CSF) and general circulation, and was thus used as a proxy for the efficacy and specificity of our central and peripheral T implants.

Statistical Analyses. ANOVAs were used to assess the effects of treatment on all measures. To avoid arbitrary value assignments to acoustic/stereotypic measures and removing birds from these analyses, we compared the PER-T, HVC-POM T, and POM-T birds in terms of these features collapsed over the final three days of treatment. Tukey's were used for post-hoc pairwise comparisons following significant omnibus ANOVAs. Correlational tests were performed using Pearson's *r*. Effects were considered significant at $P \leq 0.05$.

Results

Effects of peripheral versus central T implants. The T implants targeting the POM were localized and effective (Figure 1). Birds with T-filled cannula targeting the POM had ipsilateral POM volumes indistinguishable from the analogous side in birds that received peripheral T filled silastic implants. The contralateral POM

in POM-T birds was significantly smaller than the ipsilateral side as well as the analogous side in PER-T birds and indistinguishable from the POM of birds in the POM-NO T group. These data strongly suggest our implants were effective in inducing POM function in POM-T birds equivalent to that of PER-T birds and these implants were localized. Additionally, systemic T implants resulted in peripherally circulating concentrations of T (see below) in the range of those observed in intact birds (Alvarez-Borda and Nottebohm, 2002). The ipsilateral HVC of birds in the HVC-T group was significantly larger than the contralateral side and indistinguishable from birds in the PER-T group. Thus, the HVC T implants also appeared to be effective and localized.

Moreover, there appeared to be no leakage from the central T implants into general circulation (Figure 2). For instance, PER-T birds had larger width of the cloacal protuberance relative to all other groups (Tukey's, $p<0.05$), which were indistinguishable from one another (all comparisons, $p\geq0.50$). There was also a main effect of treatment on syrinx mass (ANOVA, $p<0.05$), such that PER-T birds had significantly larger syrinx mass than POM-T, HVC-T, and HVC-POM NO T birds ($p<0.05$). Interestingly, HVC-POM T birds were intermediate between PER-T birds and the other groups ($p=0.34$ and $0.20>p<0.94$, respectively). Nonetheless, there was no evidence of leakage of T from central implants into the general circulation (PER-T versus centrally implanted birds and empty controls, $p<0.001$; centrally-implanted birds versus empty controls, $p=0.71$; single-T implanted birds versus double-T implanted birds, $p=0.42$; individual post-hoc comparisons between PER-T and the 4 other groups, $p<0.001$; post-hoc

comparisons between the 4 other groups, $p \geq 0.63$). Lastly, PER-T birds had high circulating T concentrations (1801±159 pg/ml (Mean±SEM)) and the castrate control groups had substantially lower circulating levels (203±39 pg/ml). These T concentrations are in line with previous studies (Alvarez-Borda and Nottebohm, 2002; Alward et al., 2013).

Testosterone in the HVC regulates song stereotypy and complexity but not energy. As we have shown previously, T in the POM enhanced song rate to the levels of birds with T acting globally (PER-T versus POM-T, $p=0.41$; PER-T and POM-T versus POM-NO T and HVC-T, $p<0.05$;). Only 1or 2 birds that received T solely within their HVC or birds that did not receive T contacting their POM were observed singing at all during the course of the experiment and they only produced a few songs in total. Therefore, birds from these groups were not included in the analysis of the acoustic features of song.

T in the HVC activated the stereotypy of songs in birds that also had T in their POM to levels that were indistinguishable from birds with T acting globally (Figure 3A-B). Indeed, the stereotypy of songs produced by these two groups were about double that of birds with T solely in their POM. Measures of complexity also are regulated by T in the HVC of birds with T in their POM, but not fully (Figure 3D-E). T in the POM did not activate these complexity measures of song the levels of songs produced by PER-T birds. However, neither T in the HVC or T in the POM was able to enhance the energy or loudness of songs to that of PER-T birds; indeed, PER-T birds sang songs that were about 3 times as loud (Figure 3C).

Testosterone and singing activity modulate SCS plasticity.

HVC

Treatment significantly affected both contralateral and ipsilateral HVC volumes (Figure 4A; ANOVA, $p<0.05$ for both). HVC-T birds possessed ipsilateral HVC that was indistinguishable from PER-T birds and within HVC-T the ipsilateral side was larger than the contralateral side ($p<0.05$ for both comparisons). The ipsilateral HVC in this group was also different than the analogous side in the HVC-NO T group (Tukey's, $p<0.05$). PER-T, HVC-POM T, and POM-T birds did not differ from one another in terms of contralateral or ipsilateral HVC (Tukey's, $p \geq 0.39$ for all comparisons). For the contralateral HVC, PER-T, HVC-POM T, and POM-T birds had significantly larger HVC compared to HVC-POM NO T (Tukey's, $p<0.05$). PER-T birds had larger contralateral HVC relative to HVC-T birds ($p<0.05$). HVC-POM T and POM-T birds appeared to be different when compared to HVC-T; however, this difference was not significant (Tukey's, $p = 0.12$ and 0.09, respectively). Using Fischer's LSD tests, however, both of these differences would be considered significant. For ipsilateral HVC, PER-T, HVC-POM T and HVC-T birds were not different than one another ($p \geq 0.20$ for all comparisons). The ipsilateral HVC in HVC-POM NO T birds was smaller compared to all other groups ($p<0.05$ for all comparisons). Lastly, there was a there was a significant correlation between singing rate and the total volume of HVC (Figure 4D; $p<0.05$).

RA

Treatment significantly affected both contralateral and ipsilateral RA volumes (Figure 4B; ANOVA, $p<0.05$ for both). HVC-T birds possessed ipsilateral RA that was indistinguishable from PER-T birds and within HVC-T the ipsilateral side was larger than the contralateral side ($p<0.05$ for both comparisons). The ipsilateral RA in this group was also different than the analogous side in the HVC-NO T group (Tukey's, $p<0.05$). PER-T, HVC-POM T, and POM-T birds did not differ from one another in terms of contralateral or ipsilateral RA (Tukey's, $p \geq 0.17$ for all comparisons). For the contralateral side, PER-T, HVC-POM T, and POM-T birds had significantly larger RA compared to HVC-POM NO T (Tukey's, $p<0.05$). PER-T birds had larger contralateral RA relative to HVC-T birds ($p<0.05$). HVC-POM T and POM-T birds, however, did not differ from HVC-T birds in terms of RA volume ($p \geq 0.21$ for both comparisons). Lastly, HVC-T and HVC-POM NO T did not differ from one another for contralateral RA volume.

For ipsilateral RA, PER-T, HVC-POM T and HVC-T birds were not different than one another ($p \geq 0.20$ for all comparisons). The ipsilateral RA in HVC-POM NO T birds was smaller compared to all other groups ($p<0.05$ for all comparisons). Lastly, there was a significant correlation between singing rate and the total volume of RA (Figure 4E; $p<0.05$).

Area X

Treatment significantly affected both contralateral and ipsilateral Area X volumes (Figure 4C; ANOVA, $p<0.05$ for both). HVC-T birds possessed ipsilateral Area X

that was statistically indistinguishable from PER-T birds based on Tukey's ($p=0.15$). The ipsilateral Area X in this group was also different than the analogous side in the HVC-NO T group (Tukey's, $p<0.05$). PER-T, HVC-POM T, and POM-T birds did not differ from one another in terms of contralateral or ipsilateral Area X (Tukey's, $p \geq 0.14$ for all comparisons). For the contralateral Area X, PER-T, HVC-POM T, and POM-T birds had significantly larger Area X compared to HVC-POM NO T (Tukey's, $p<0.05$). PER-T birds had larger contralateral Area X relative to HVC-T birds ($p<0.05$). HVC-POM T and POM-T birds, however, did not differ from HVC-T birds in terms of Area X volume volume ($p \geq 0.48$ for both comparisons). Lastly, HVC-T and HVC-POM NO T did not differ from one another for contralateral Area X volume ($p=0.27$).

For ipsilateral Area X, PER-T, HVC-POM T, POM-T, and HVC-T birds were not different than one another (Tukey's, $p \geq 0.18$ for all comparisons). The ipsilateral Area X in HVC-POM NO T birds was smaller compared to all other groups ($p<0.05$ for all comparisons). Lastly, there was a there was a significant correlation between singing rate and the total volume of Area X (Figure 4F; $p<0.05$).

Testosterone and singing affect the differentiation of new neurons in HVC but testosterone is required for the recruitment of new neurons. One bird from the HVC-POM T group had damage within HVC that precluded the counting of DCX and was thus not included in the analysis. The numbers of DCX round and fusiform cells in control regions (lateral to HVC and ventral to HVC) were unaffected by testosterone treatment ($p \geq 0.24$ for all tests). Testosterone

treatment had significant effects on both contralateral and ipsilateral incorporation of new neurons (Figure); ANOVA, $p<0.05$ for both). Post-hoc tests revealed PER-T birds had substantially more fusiform cells incorporated into contralateral HVC compared to all of the other groups (Tukey's, $p<0.05$ for all comparisons to PER-T). However, PER-T, HVC-POM T, and HVC-T birds were all indistinguishable from one another in terms of the number of fusiform cells added to ipsilateral HVC (Tukey's, $p\geq0.21$ for all comparisons). POM-T birds had fewer fusiform cells in the ipsilateral HVC compared to PER-T birds (Tukey's, $p<0.05$) but were statistically indistinguishable compared to HVC-POM T, HVC-T, and HVC-POM NO T birds (Tukey's, $p\geq0.14$ for all comparisons).

Testosterone also significantly affected the number of DCX round cells in the contralateral and ipsilateral HVC (Figure 5 C-D; Between-subjects ANOVAs, $p<0.05$ for both). PER-T birds had more round cells in the contralateral HVC compared to HVC-T and HVC-POM NO T birds (Tukey's, $p<0.05$ for both comparisons) but not different when compared to HVC-POM T and POM-T birds (Tukey's, $p\geq0.14$ for both comparisons). HVC-POM T and POM-T birds were not different compared to one another or when compared to HVC-T and HVC-POM NO T birds (Tukey's, $p\geq0.55$ for all comparisons). On the ipsilateral side, PER-T birds had more round cells than HVC-POM NO T (Tukey's, $p<0.05$) but were no different when compared to HVC-POM T, POM-T, or HVC-T birds (Tukey's, $p\geq0.45$ for all comparisons). As above, HVC-POM T and POM-T birds were no different than PER-T birds but also no different than HVC-T and HVC-POM NO T (Tukey's, $p\geq0.19$ for all comparisons).

There was a positive correlation between song rate and the number of round cells in the contralateral HVC (Figure 6; $p<0.05$). This relationship was not observed for total round cells ($p=0.14$) or ipsilateral round cells ($p=0.41$) in HVC. There was also a positive correlation for the total number of fusiform cells and the number of fusiform cells in the contralateral HVC ($p<0.05$ for both). This was not observed for the ipsilateral HVC ($P=0.17$).

However, it appeared that some birds were showing a systematically different relationship between song rate and DCX cells in HVC compared to the others. Specifically, when we plotted birds that sang without globally circulating T (i.e., HVC-POM T and POM-T birds) separately, a significant negative correlation emerged between song rate and the total number of DCX round cells in HVC ($p<0.05$). For contralateral and ipsilateral round cells this relationship was also negative but was not significant ($p=0.11$ and 0.13, respectively). There was no correlation for these birds between song rate and DCX fusiform cells ($p\geq0.52$ for all tests).

Discussion

Steroid hormones can have substantial effects on behavior as well as the neural substrates that regulate these behaviors. Here we have demonstrated that different features of a single behavior can be regulated by the actions of hormones in distinct regions of the brain. Moreover, we have shown that hormones can regulate neuroplasticity via different mechanisms: by acting

directly at targets sites or via activity driven mechanisms, perhaps even due to transsynaptic processes.

T in the HVC enhances song stereotypy and plays a role in modulating song complexity but not song energy

We replicated our previous work showing that T in the POM is sufficient to activate singing rate but not features of song such as song stereotypy and song energy (Alward et al., 2013). Based on work by Meitzen and colleagues we hypothesized that T needs to act in the HVC to enhance song stereotypy to levels present in PER-T birds (Meitzen et al., 2007; Meitzen et al., 2009; Meitzen and Thompson, 2008). To test this we implanted T in the HVC of birds that had T in the POM. Indeed, we found that T in the HVC was sufficient in activating all of the features of song stereotypy that were absent in birds with T only in their POM. We also showed that T in the HVC partially plays a role in regulating the complexity of song, as this song feature was activated to intermediate levels. These data suggest that T in the HVC plays a substantial role in regulating acoustic features of song and that complexity may be regulated by T acting at multiple levels of the song system, such as RA or the syrinx.

Contrarily, T in the HVC did not enhance the energy of songs: the HVC-POM T and POM-T birds were indistinguishable from one another and sang songs that were three times quieter than the PER-T birds. These results are not surprising, however, considering most research on the regulation of the amplitude of vocalizations centers on respiratory activity playing an integral role (Plummer and

Goller, 2008; Franz and Goller, 2002; Podos, 1997; Wild, Goller, and Suthers, 1998). This is intriguing considering that nuclei involved in coordinating respiratory activity during singing, such as the nucleus Ram and Pam in the hindbrain densely express androgen receptors (Gahr and Wild, 1997). Thus, it appears that the amplitude of songs is more directly related to peripheral mechanisms that could be regulated by actions of T in the hindbrain.

The actions of T in the regulation of SCS neuroplasticity are multileveled and possibly interactive.

Previous studies have shown that sex steroid hormones have robust effects on the plasticity and function of various brain regions. For instance work by Brenowitz and Lent (2002) have shown that T actions in HVC can enhance the volumes of the ipsilateral HVC as well as the ipsilateral side of the downstream nuclei RA and Area X. Other studies have shown that the mere production of a T-driven behavior, birdsong, can cause volumetric and neural changes in the SCS as well (Alvarez-Borda and Nottebohm, 2002; Alward et al., 2013; Chapter 4). Here we provide evidence in support of both of these hypotheses. T implanted in HVC was able to enhance the volume of the ipsilateral HVC. Singing, presumably on its own, was able to enhance the volumes of HVC, RA, and Area X, as our lab has shown in recent work (Alward et al 2013). Interestingly, the effects of T acting directly in HVC to enhance volume and the effects of singing did not appear to be additive. This is not surprising, however. For example, in our previous work birds implanted with T in their POM and birds with T acting globally did not differ in the size of their SCS nuclei (Alward et al., 2013). This does not

eliminate the possibility that T and singing activity interact on some other level, such as modulating neural function or morphology at lower levels.

We also showed that the direct action of T in HVC promotes new neurons to be incorporated into HVC and another factor, perhaps singing activity, modulates the incorporation of new neurons into HVC. Work by Alvarez-Borda and Nottebohm (2002) had alluded to the possibility that gonadal steroids and singing activity can independently and additively modulate the incorporation of new neurons into HVC. Results from the current study indicate that T acting directly in HVC modulates this new neuron recruitment and singing activity may also play a role in the incorporation of these new neurons into HVC.

Importantly, we replicated the work by Li and colleagues (2000), that singing activity positively correlates with the incorporation of new neurons into HVC. However, there did not appear to be separate, additive effects of T and singing activity on the incorporation of new neurons into HVC. In fact, T in HVC caused more new neurons to be incorporated into HVC than birds with T in their POM and T in their HVC and POM, based on a statistical difference between HVC-T birds and HVC-POM NO T birds for ipsilateral round and fusiform cells that was not present in HVC-POM T and POM-T birds. However, these results may partially be explained by the negative correlation observed here for song rate and DCX round cells in the HVC of HVC-POM T and POM-T birds. Indeed, these results are in line with the results obtained by Pytte et al. (2012), showing that for gonadally-intact male zebra finches, there was a slight negative correlation between song rate and new neurons added to HVC. This negative correlation

between singing activity and new neurons in HVC was not observed for PER-T birds, suggesting differential effects of T and singing on new neuron incorporation as a function of where else in the brain T is acting and for how long the bird has been singing (PER-T stimulates singing within about 3 days while POM-T takes about 7-9 days, Alward et al., 2013).

We also saw a slight increase in the syrinx mass of HVC-POM T birds. This effect was most likely not due to perihperally circulating levels of T given the results obtained for POM volumes, CP width, and measures or peripherally-circulating T. Therefore, these effects could be due to transsynaptic effects of T in HVC. Interestingly though, this effect on the syrinx was not observed for birds with T solely in their HVC or with birds with T solely in their POM, suggesting this effect is not driven solely by T or singing activity. Therefore, this slight increase in mass of the syrinx in the HVC-POM T birds could be an effect driven by T in HVC but the actual use of the syrinx—i.e., singing—could be permissive for this increase in mass to occur. Transsynaptic effects of T in HVC on RA were observed in the current study and in work by Lent and Brenowitz (2002), so it is possible these effects proceed downstream to the syrinx if the bird is singing.

Conclusion: T has pleiotropic effects in the regulation of a single, complex learned behavior and its underlying neural architecture

T's role in the regulation of sexually-motivated behaviors has been investigated for decades. Its influence on cognitive functions and non-redundant actions is less understood. We have shown that T can regulate multiple, distinct features of

a single behavior, birdsong, and can affect the plasticity of the circuit that regulates it in direct and indirect ways, depending on where it is acting in the brain. However, it seems clear that T in the HVC does not regulate the energy of song and is only partially involved in regulating the complexity of song. Future studies will aim to elucidate where else in the brain as well as the periphery (e.g. the hindbrain or respiratory muscles) T is exerting its influence on these and other components of song.

Figure Legend:

Figure 1- Graphs show the effects of T implants on the ratio POM volume of the hemisphere implanted with T (ipsilateral=ipsi) over the opposite side (contralateral-contra) and the volumes of the ipsi and contra sides. Bars represent Mean±Standard error of the mean (SEM). Letters over individual bars indicate if levels of the independent variable are different from one another—the same letter indicates no difference and different letters indicate a difference. Differences were considered significant at $P \leq 0.05$.

Figure 2- The effects of treatment on two peripheral measures of T action: A) cloacal protuberance (CP) width and B) syrinx mass. Letters over individual bars indicate if levels of the independent variable are different from one another—the same letter indicates no difference and different letters indicate a difference. Differences were considered significant at $P \leq 0.05$.

Figure 3- The effects of T implant treatment on various acoustic measures (described in the Appendix). Bars represent Mean±SEM. Letters over individual bars indicate if levels of the independent variable are different from one another—the same letter indicates no difference and different letters indicate a difference. Differences were considered significant at $P \leq 0.05$.

Figure 4- A-C shows the effects of T implants on the volume of the SCS nuclei in the hemisphere implanted with T (ipsilateral=ipsi) versus the opposite side (contralateral=contra). Bars represent Mean±SEM. Letters over individual bars indicate if levels of the independent variable are different from one another—the

same letter indicates no difference and different letters indicate a difference. Lower-case letters indicate the comparisons were made between the groups relative to the contralateral side; Upper-case letters indicate the comparisons were made between the groups relative to the ipsilateral side. Differences were considered significant at $P \leq 0.05$. D-F shows the correlations between song rate and the volume of the various SCS.

Figure 5- The effects of treatment on the number of DCX-immunoreactive cells in HVC Panels A-B show the number of fusiform cells in the contralateral (contra) and ipsilateral (ipsi) implant site, respectively. Panels C-D show the number of round cells in the contralateral (contra) and ipsilateral (ipsi) implant site, respectively. Bars represent Mean±SEM. Letters over individual bars indicate if levels of the independent variable are different from one another—the same letter indicates no difference and different letters indicate a difference.

Figure 6- The relationship between song rate and the number of DCX-immunoreactive cells in HVC.

Figures

Figure 1

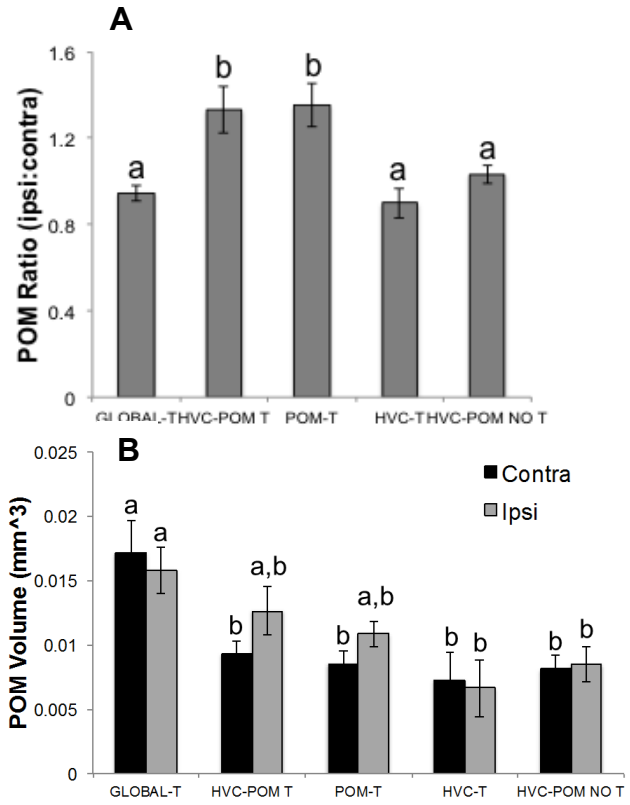


Figure 2

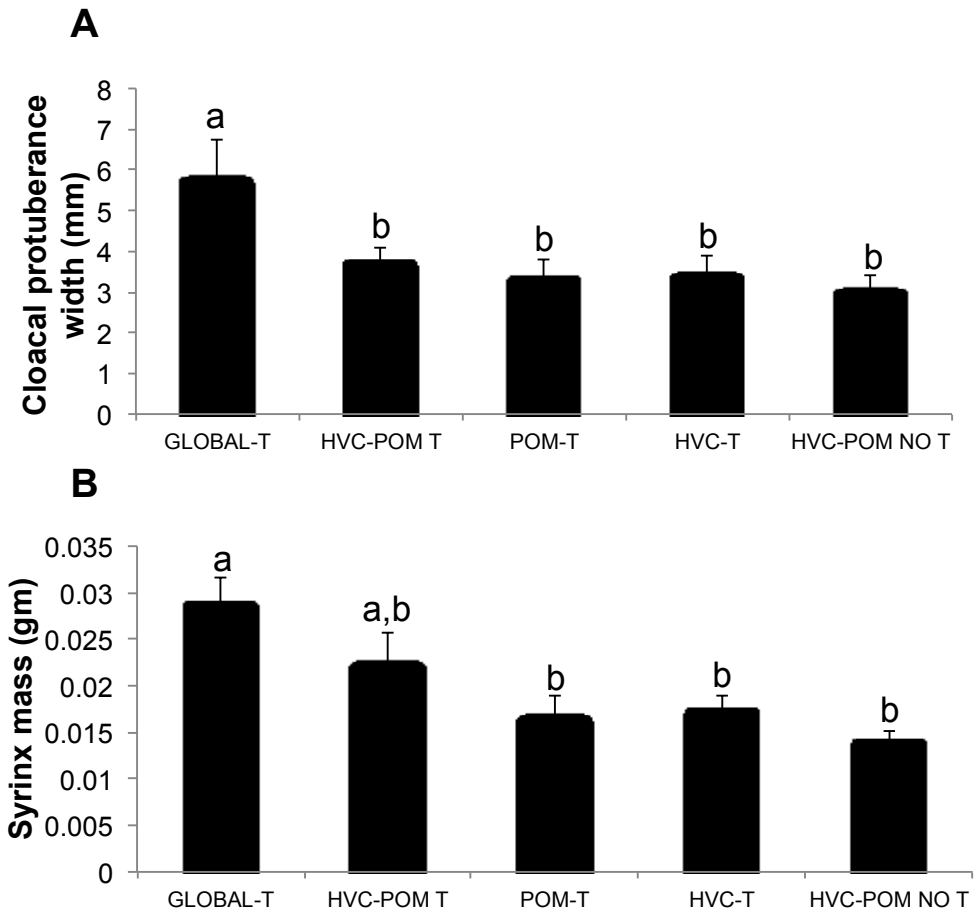

A
Cloacal protuberance width (mm)
8
7
6
5
4
3
2
1
0
a
b
b
b
b
GLOBAL-T
HVC-POM T
POM-T
HVC-T
HVC-POM NO T
B
Syrinx mass (gm)
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
a
a,b
b
b
b
GLOBAL-T
HVC-POM T
POM-T
HVC-T
HVC-POM NO T

Figure 3

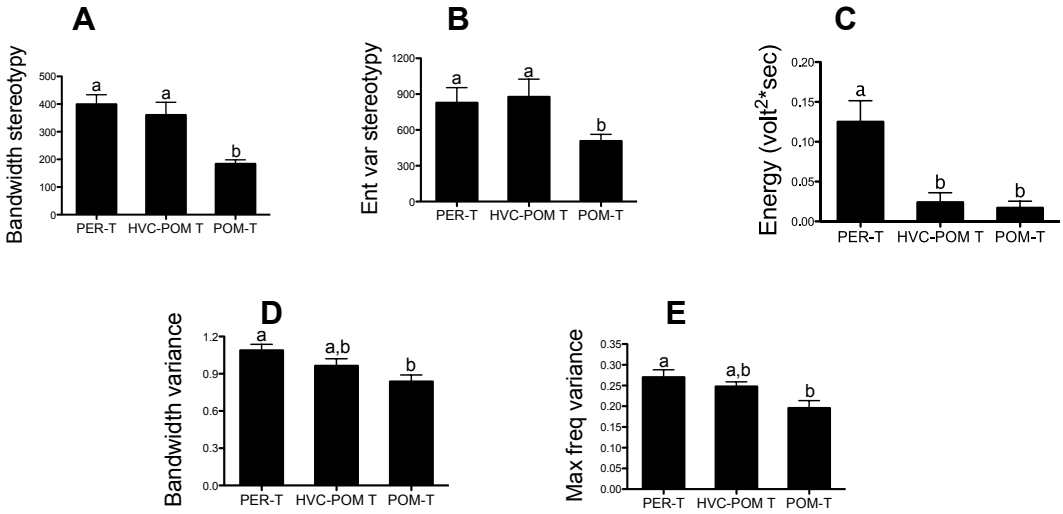

A
Bandwidth stereotypy
500
400
300
200
100
0
a
a
b
PER-T
HVC-POM T
POM-T
B
Ent var stereotypy
1200
900
600
300
0
a
a
b
PER-T
HVC-POM T
POM-T
C
Energy (volt2*sec)
0.20
0.15
0.10
0.05
0.00
a
b
b
PER-T
HVC-POM T
POM-T
D
Bandwidth variance
1.2
0.9
0.6
0.3
0.0
a
a,b
b
PER-T
HVC-POM T
POM-T
E
Max freq variance
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
a
a,b
b
PER-T
HVC-POM T
POM-T

Figure 4

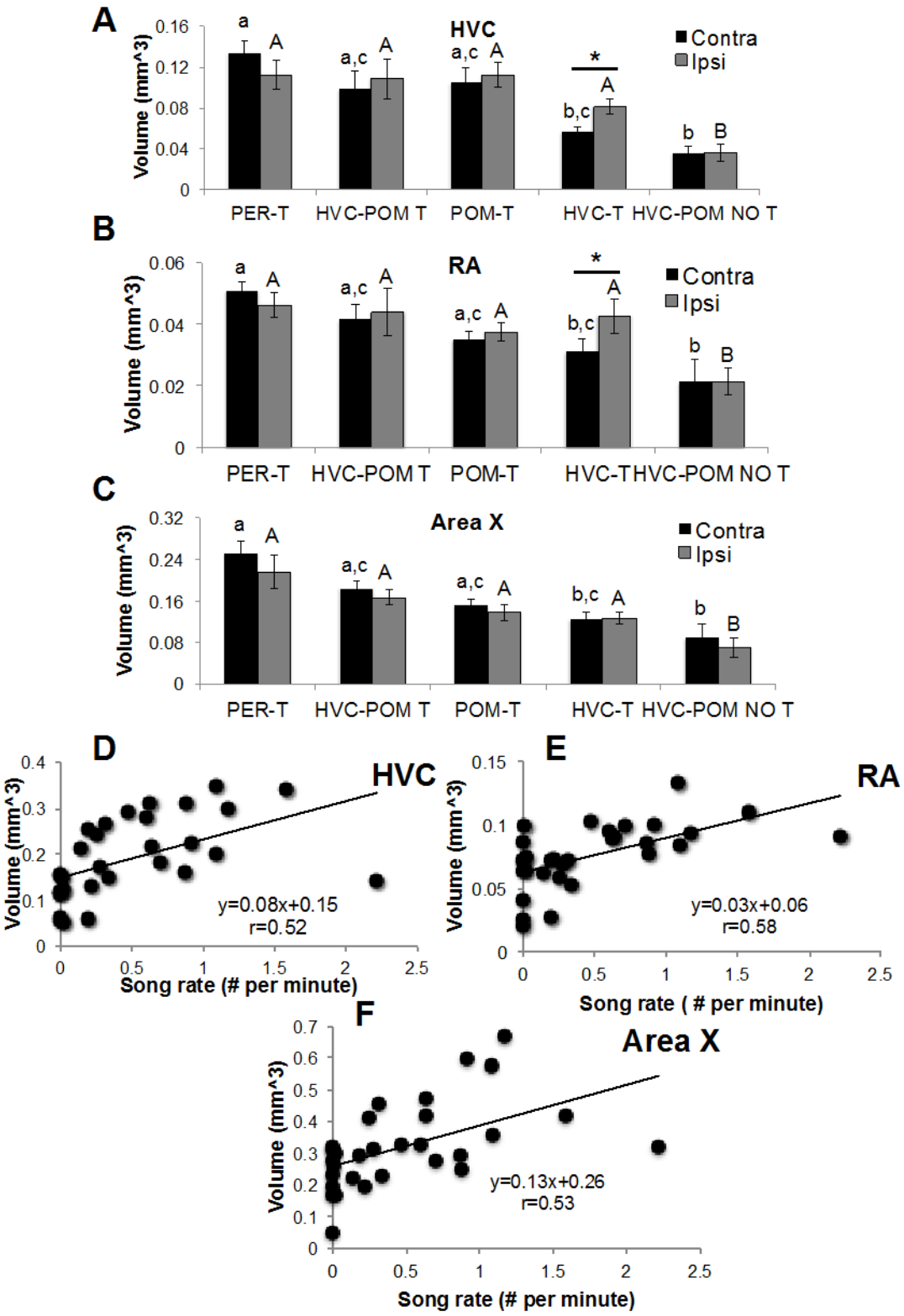

A
HVC
Contra
Ipsi
Volume (mm^3)
0.16
0.12
0.08
0.04
0
a
A
a,c
A
a,c
A
*
b,c
A
b
B
PER-T
HVC-POM T
POM-T
HVC-T
HVC-POM NO T
B
RA
Contra
Ipsi
Volume (mm^3)
0.06
0.04
0.02
0
a
A
a,c
A
a,c
A
*
b,c
A
b
B
PER-T
HVC-POM T
POM-T
HVC-T
HVC-POM NO T
C
Area X
Contra
Ipsi
Volume (mm^3)
0.32
0.24
0.16
0.08
0
a
A
a,c
A
a,c
A
b,c
A
b
B
PER-T
HVC-POM T
POM-T
HVC-T
HVC-POM NO T
D
HVC
Volume (mm^3)
0.4
0.3
0.2
0.1
0
y=0.08x+0.15
r=0.52
0
0.5
1
1.5
2
2.5
Song rate (# per minute)
E
RA
Volume (mm^3)
0.15
0.1
0.05
0
y=0.03x+0.06
r=0.58
0
0.5
1
1.5
2
2.5
Song rate (# per minute)
F
Area X
Volume (mm^3)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
y=0.13x+0.26
r=0.53
0
0.5
1
1.5
2
2.5
Song rate (# per minute)

Figure 5

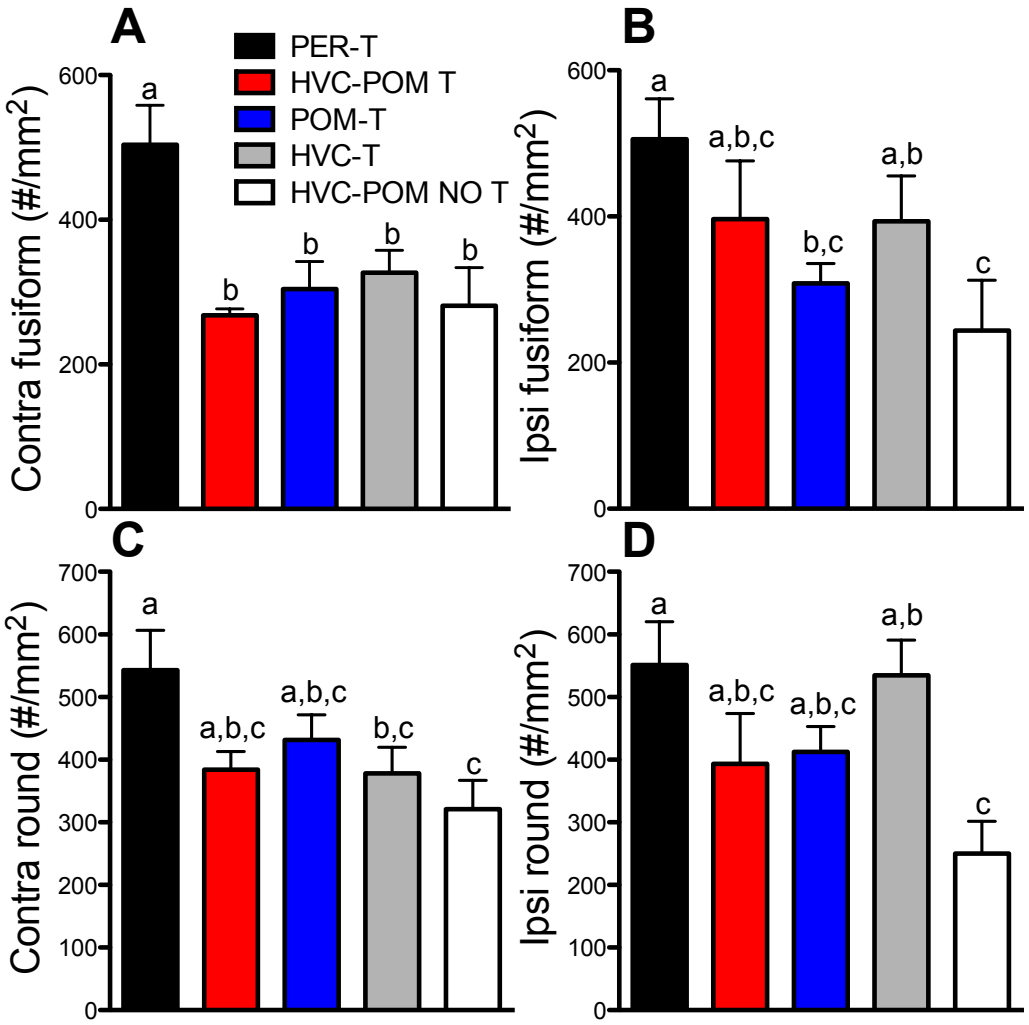
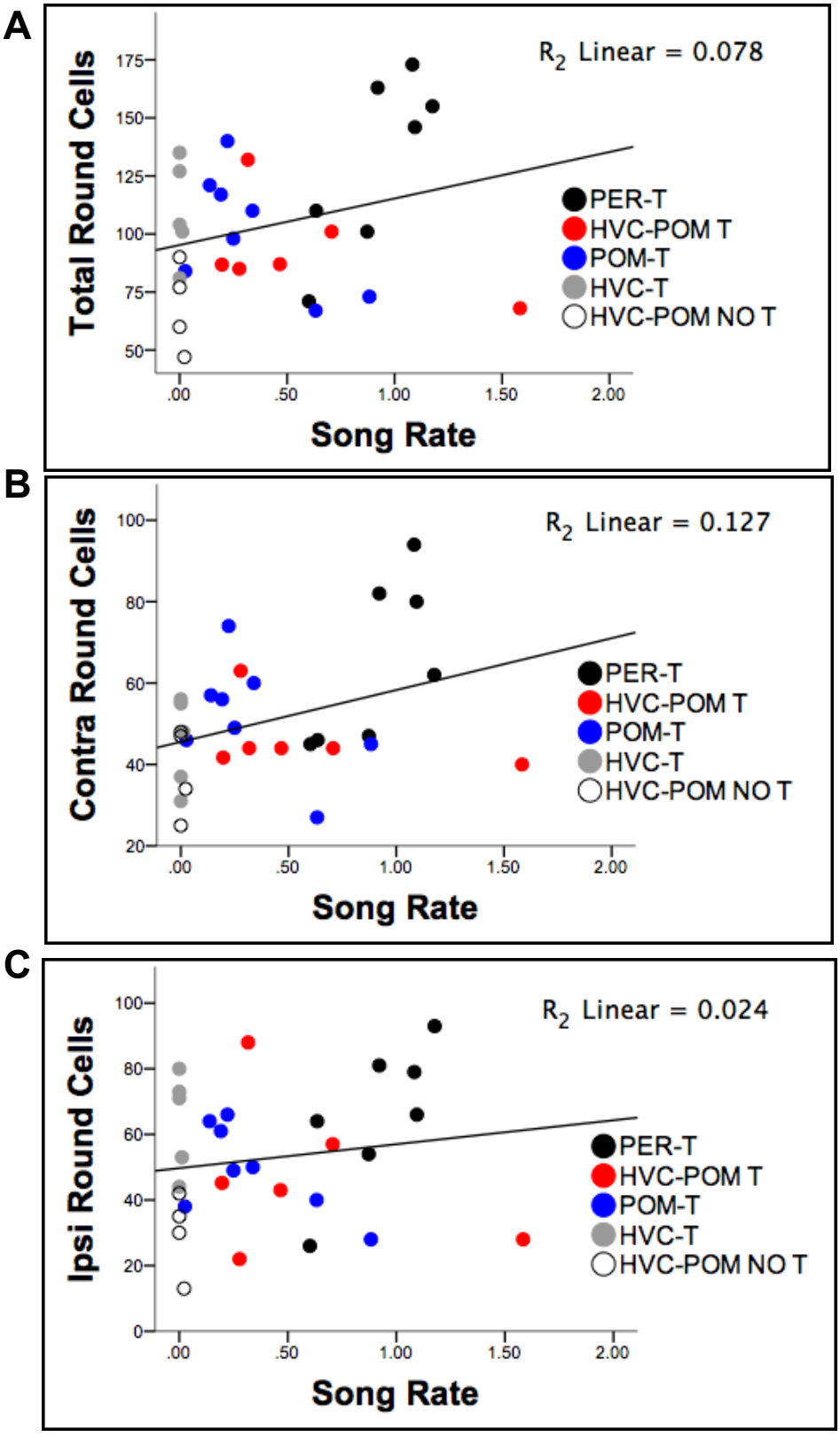
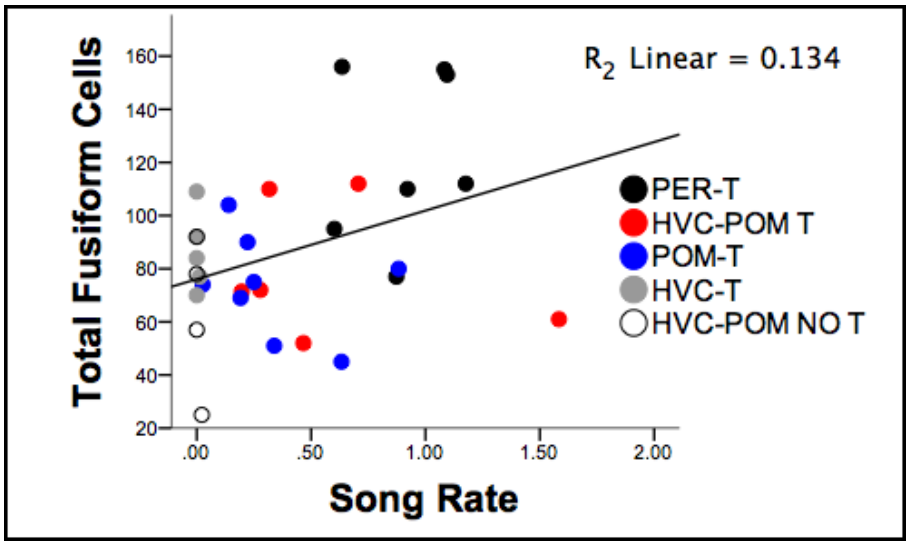

A
B
C
D
PER-T
HVC-POM T
POM-T
HVC-T
HVC-POM NO T
Contra fusiform (#/mm2)
Ipsi fusiform (#/mm2)
Contra round (#/mm2)
Ipsi round (#/mm2)
0
100
200
300
400
500
600
700
a
b
a,b,c
b,c
c
a,b

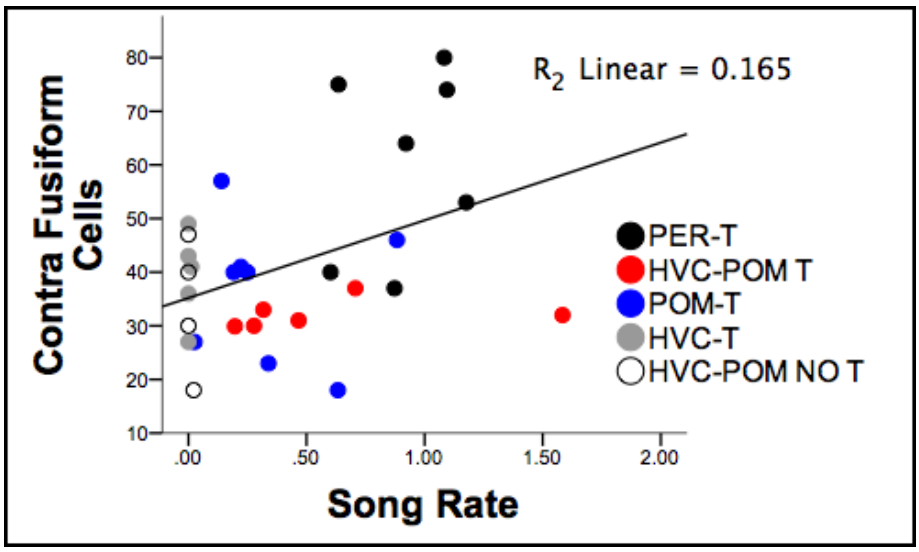
Figure 6


A
Total Round Cells
175
150
125
100
75
50
R_2 Linear = 0.078
PER-T
HVC-POM T
POM-T
HVC-T
HVC-POM NO T
.00
.50
1.00
1.50
2.00
Song Rate
B
Contra Round Cells
100
80
60
40
20
R_2 Linear = 0.127
PER-T
HVC-POM T
POM-T
HVC-T
HVC-POM NO T
.00
.50
1.00
1.50
2.00
Song Rate
C
Ipsi Round Cells
100
80
60
40
20
0
R_2 Linear = 0.024
PER-T
HVC-POM T
POM-T
HVC-T
HVC-POM NO T
.00
.50
1.00
1.50
2.00
Song Rate

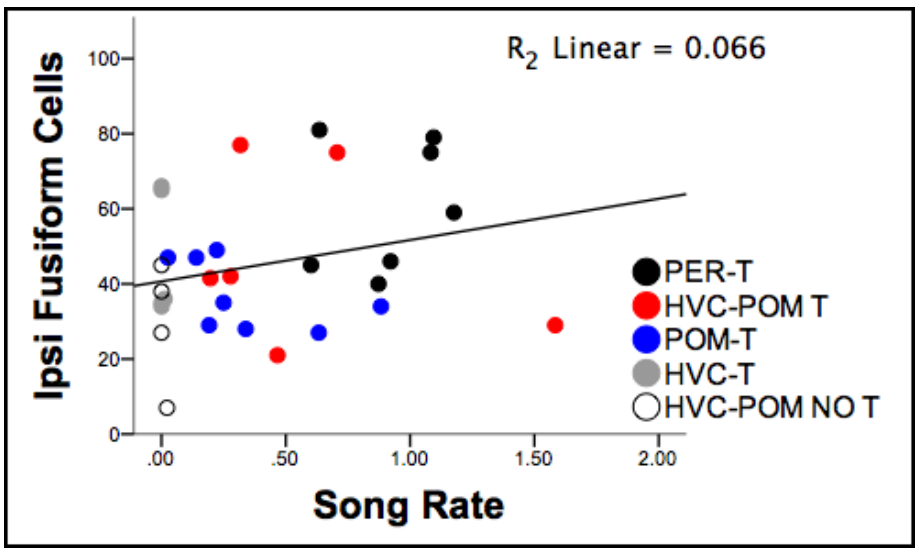
D


Total Fusiform Cells
160
140
120
100
80
60
40
20
.00
.50
1.00
1.50
2.00
Song Rate
R2 Linear = 0.134
PER-T
HVC-POM T
POM-T
HVC-T
HVC-POM NO T

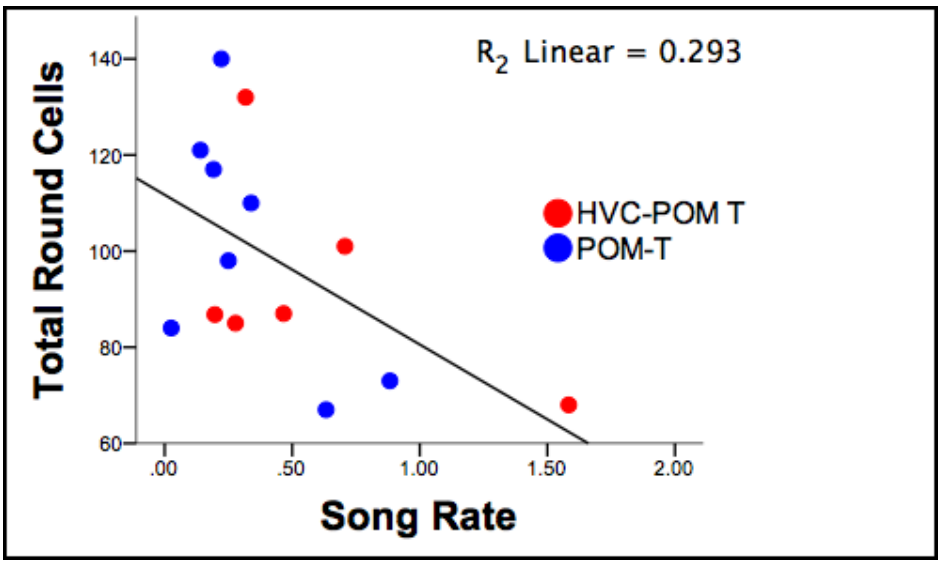
E


Contra Fusiform Cells
80
70
60
50
40
30
20
10
.00
.50
1.00
1.50
2.00
Song Rate
R2 Linear = 0.165
PER-T
HVC-POM T
POM-T
HVC-T
HVC-POM NO T

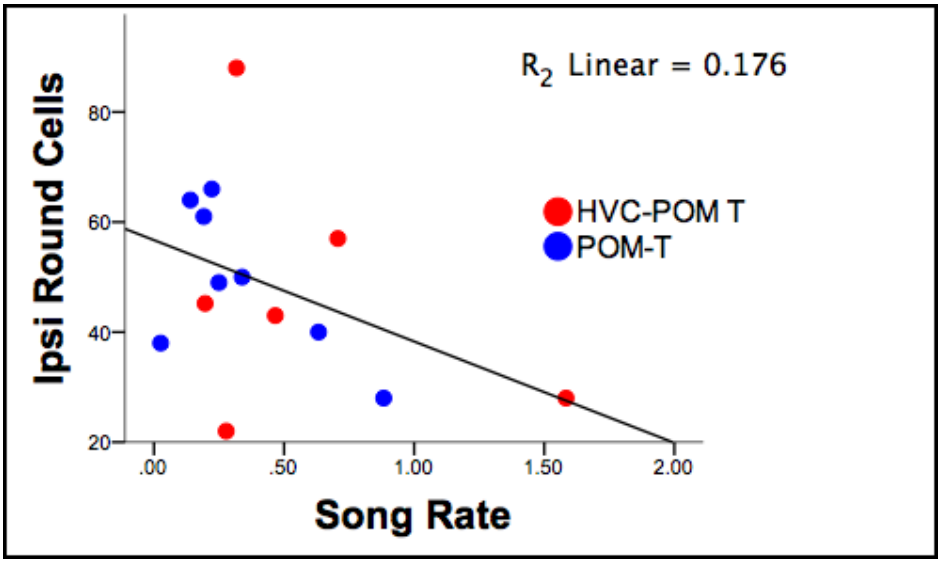
F


Ipsi Fusiform Cells
100
80
60
40
20
0
.00
.50
1.00
1.50
2.00
Song Rate
R2 Linear = 0.066
PER-T
HVC-POM T
POM-T
HVC-T
HVC-POM NO T

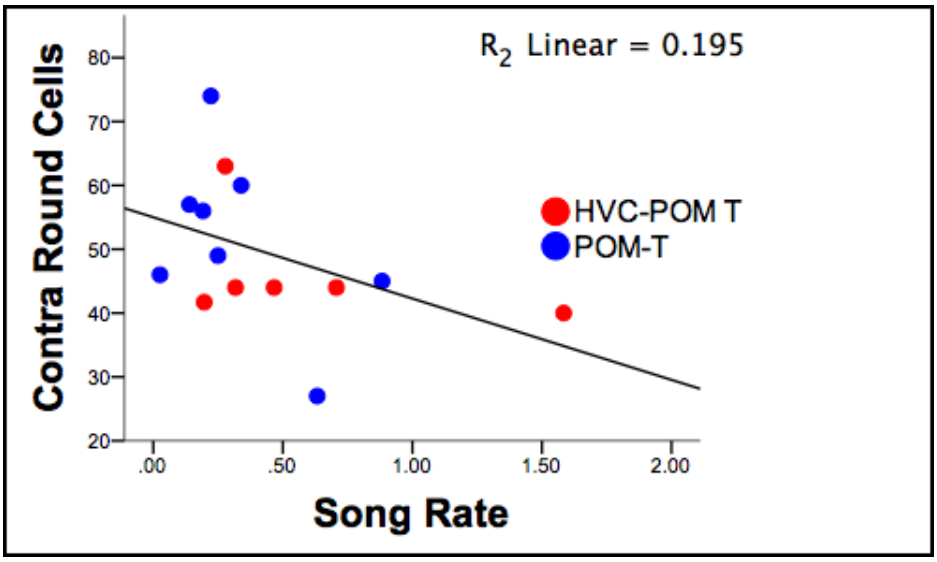
G


R_2 Linear = 0.293
Total Round Cells
140
120
100
80
60
.00
.50
1.00
1.50
2.00
Song Rate
HVC-POM T
POM-T

H


R_2 Linear = 0.176
Ipsi Round Cells
80
60
40
20
.00
.50
1.00
1.50
2.00
Song Rate
HVC-POM T
POM-T

I


R_2 Linear = 0.195
Contra Round Cells
80
70
60
50
40
30
20
.00
.50
1.00
1.50
2.00
Song Rate
HVC-POM T
POM-T

Chapter 6: Investigating the effects of testosterone in the HVC on trills in canary song

Introduction

In chapter 4, it was established that T in the HVC of castrated male canaries who also had T implanted in the POM increased the stereotypy of songs produced and to some extent though not completely they produced songs with an increase in complexity. This raised the question of what specific features of the canary song may be regulated by the actions of T in HVC? For instance, male canaries, like many other songbirds, produce vocalizations called trills throughout their songs. Trills are groups of the same notes sung in rapid succession, the production of which appears to be under strong evolutionary pressures among many songbirds and appear to be T dependent (Podos, 1997). In line with this, male canaries produce a special type of trill called 'special syllables' or 'sexy syllables', which have a two-note structure with a very fast repetition rate and are especially sexually-stimulating to females as assessed based on their ability to elicit a copulation solicitation display (Vallet et al., 1998; Vallet and Kreutzer, 1995). Moreover, the rate of production of these sexy syllables is higher during the breeding season than the non-breeding season (Voigt and Leitner, 2008). Lastly, Halle, Gahr, and Kreutzer (2003) showed that unilateral lesions to HVC, in addition to causing perturbations to overall syllable acoustic structure, caused disruptions in the acoustic features of sexy syllables.

Whether T acting in HVC specifically activates these sexy syllables is unclear. Hence, the goal of the current study is to investigate the role of T in the HVC in the regulation of trills as well as the sexy syllables. This study will use the

song recordings made from the study in which T was implanted in the POM and HVC of canaries in chapter 4 but only focus on the production and acoustic features of trills (i.e., only PER-T, HVC-POM T, and POM-T birds were analyzed).

Methods

Materials and Methods

Animals and pre-experimental manipulations. See preceding chapter.

Experimental groups and stereotaxic implantation. See preceding chapter.

Song trill features. Each day video and audio recordings were made from 800h to 930h (lights on at 800 h), 1300h to 1430h, and 1600-1730h. We quantified song features as mentioned in the Appendix collapsed on the final two days of treatment using the sound analysis program Avisoft and from these features determined trill stereotypy (Alward et al., 2013; Meitzen et al., 2007). On each of these days, we looked specifically within song bouts and analyzed trills within continuous singing of five minutes, which on average allowed for analysis of 10 trills of each type on each day analyzed, or the maximum number of trills could be quantified (some birds sang trills very infrequently). A trill is defined as a rapidly sung sequence of repeated notes (Podos, 1997). As mentioned above, trills were defined as vocalizations including identical syllables being sung in rapid succession. As mentioned above, male canaries also produce sexy syllables, which are defined as having a two-note structure and fast repetition rate (Podos, 1997; Vallet et al., 1998; Vallet and Kreutzer, 1995). Unique trill types, as is the case with any unique vocalization, are distinguishable from other

unique trill types based on specific morphological features on the spectrogram and were determined using visual inspection. Using Avisoft we were able to save images of each trill and quantify features of all of these trills; we quantified the same features for trills as we did for whole songs. Images of each trill type were saved into Powerpoint and all acoustic parameters were analyzed per each trill and also collapsed into an overall trill value. This set of collapsed trill features were determined using the specific values for each trill type and then collapsing across all the different types of trills. This collapsed measure also included the sexy syllables. While we were analyzing the various trill types, it became apparent that most birds produced three particular types of trills: one trill included simple notes sung in extremely rapid secession (Figure 2); two other trills had the structure and syllable repetition rate characteristic of two different sexy syllable types (Figures 3 and 4; Podos, 1997; Vallet et al., 1998; Vallet and Kreutzer, 1995). We designated these as trill α, β, and γ, respectively. These trills were analyzed separately in addition to the overall trill analysis.

Brain collection and verification of implants and castrations. See preceding chapter.

Brain and serum analyses. See preceding chapter.

POM volume reconstruction. See preceding chapter.

Statistical Analyses. See preceding chapter.

Results

Peripheral and central implants of T were efficacious. See preceding chapter.

Effects of Testosterone in the HVC on the acoustic features of trills and sexy syllables For the collapsed trill values, multiple song features were affected (Figure 1). For instance, PER-T birds sang trills with a higher repetition rate (notes/trills/second) than POM-T birds ($P<0.05$) while HVC-POM T birds had trill repetition rates between these two groups (HVC-POM T versus PER-T, $p=0.16$; versus POM-T, $p=0.71$). This pattern was present for bandwidth as well (PER-T versus both groups, $p<0.05$; HVC-POM T versus PER-T, $p=0.10$; versus POM-T, $p=0.47$). PER-T birds also sang overall louder trills than the other two groups ($p<0.05$), which did not differ from one another ($p=0.98$).

For the three separate trill types, α, β, and γ, a number of features were affected by treatment. For trill alpha, PER-T birds on average sang more of these trills per minute compared to HVC-POM T and POM-T birds ($p<0.05$). PER-T birds had more syllables within trills compared to POM-T birds ($p<0.05$), but did not differ compared to HVC-POM T ($p=0.42$); HVC-POM T birds did not differ compared to POM-T birds ($p=0.28$). A similar pattern was present for syllable repetition rate within trills (PER-T versus POM-T, $p<0.05$; HVC-POM T versus PER-T and POM-T, $p=0.32$ and 0.24, respectively).

For trill beta, a sexy syllable, treatment affected multiple acoustic features. For instance, PER-T birds produced the loudest beta trills compared to HVC-POM T and POM-T ($p<0.05$), which did not differ ($p=0.99$). PER-T birds produced beta trills with higher maximum frequency variance compared to POM-T birds

($p<0.05$). HVC-POM T birds did not differ from PER-T birds or POM-T birds in regards to this acoustic variable ($p=0.26$ and 0.43, respectively). PER-T birds sang more stereotypic trills than both groups ($p<0.05$), which did not differ ($p>0.9$).

For trill gamma, PER-T birds produced more of these trills per minute and with higher bandwidth stereotypy compared to POM-T ($p<0.05$). PER-T birds and HVC-POM T birds were not different in terms of these variables, although there was a tendency for PER-T birds to have higher values ($p=0.08$ and 0.10, respectively).

Discussion

Recently, evidence has begun to accumulate indicating that T acts in multiple regions of the songbird brain to regulate specific features of song and neuroplasticity. For instance, T may act in one region to regulate the motivation to sing while acting in another region to regulate the stereotypy or loudness of song. In other words, T has non-redundant actions when regulating a single behavior (Arnold, 1981). The current study takes this avenue of investigation a step further by assessing the role of T in the songbird brain in the regulation of specific vocalizations, trills, including the sexy syllables, a physiologically demanding trill that is highly attractive to females (Podos, 1997; Suthers et al., 2004; Vallet et al., 1998; Vallet and Kreutzer, 1995).

In the current study we demonstrated that T in the HVC regulates different features of canary vocalizations. For multiple features of trill stereotypy, complexity, and repetition rate T in the HVC played a partial role or was either ineffective. Several interpretations of these data can be made. For instance, it is well known that the syrinx plays an integral role in generating trills (Chapter 3; Podos, 1997). Indeed, multiple studies have described the morphological changes that the syrinx must undergo to generate trills including sexy syllables has been well characterized and demonstrate how both sides of the syrinx are involved (Podos, 1997; Suthers et al., 2012, 2004). Especially relevant to the present study, androgenic action enhances the activity of acetylcholine esterase and increases the number of acetylcholine receptors (Luine, Nottebohm, and Harding 1980) and the results of chapter 3 indicate that AR at the syrinx are involved in regulating the complexity of both songs and trills. Therefore, T in both the HVC and the syrinx may be involved in regulating these trills and T in the HVC partially enhanced these trills features but the syrinx was not primed with androgens.

T in the HVC plays a critical role in regulating multiple levels of canary song, such as whole songs (Chapter 5) and individual vocalizations like trills and sexy syllables. Given that for HVC-POM T birds almost all of these trill features were only activated to intermediate levels or not different compared to POM-T birds, it seems plausible that T acting in other areas is required to fully activate these features. The syrinx is another candidate site but other brain regions like RA (Margoliash, 1997b) or respiratory regions in the hindbrain like Ram and Pam

could also be critical (Bernard et al., 1999; Hartley, 1990; Hartley and Suthers, 1989). In the end, this study highlights the important role steroid hormones play in coordinating the distinct features of a complex learned behavior into a single, functional response.

Figure Legend

Figure 1- Graphs depict the effects of T treatment on all trill types collapsed into overall trill values. The acoustic features are described in the Appendix. Bars represent Mean±SEM. Letters over individual bars indicate if levels of the independent variable are different from one another—the same letter indicates no difference and different letters indicate a difference. Differences were considered significant at $P \leq 0.05$.

Figures 2 through 4- These figures depict the effects of T treatment on distinct categories of trill types produced. Trills β and γ are considered to be sexy syllables (Vallet et al., 1998). Bars represent Mean±SEM. Letters over individual bars indicate if levels of the independent variable are different from one another—the same letter indicates no difference and different letters indicate a difference. Differences were considered significant at $P \leq 0.05$.

Figures

Figure 1: Trills collapsed

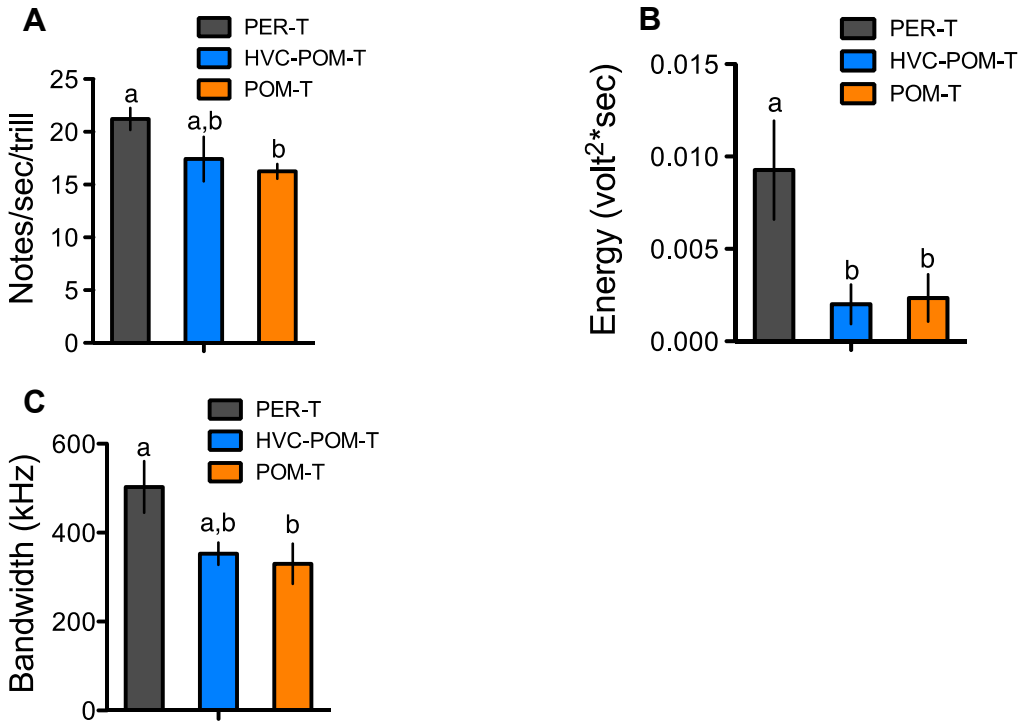


Figure 2

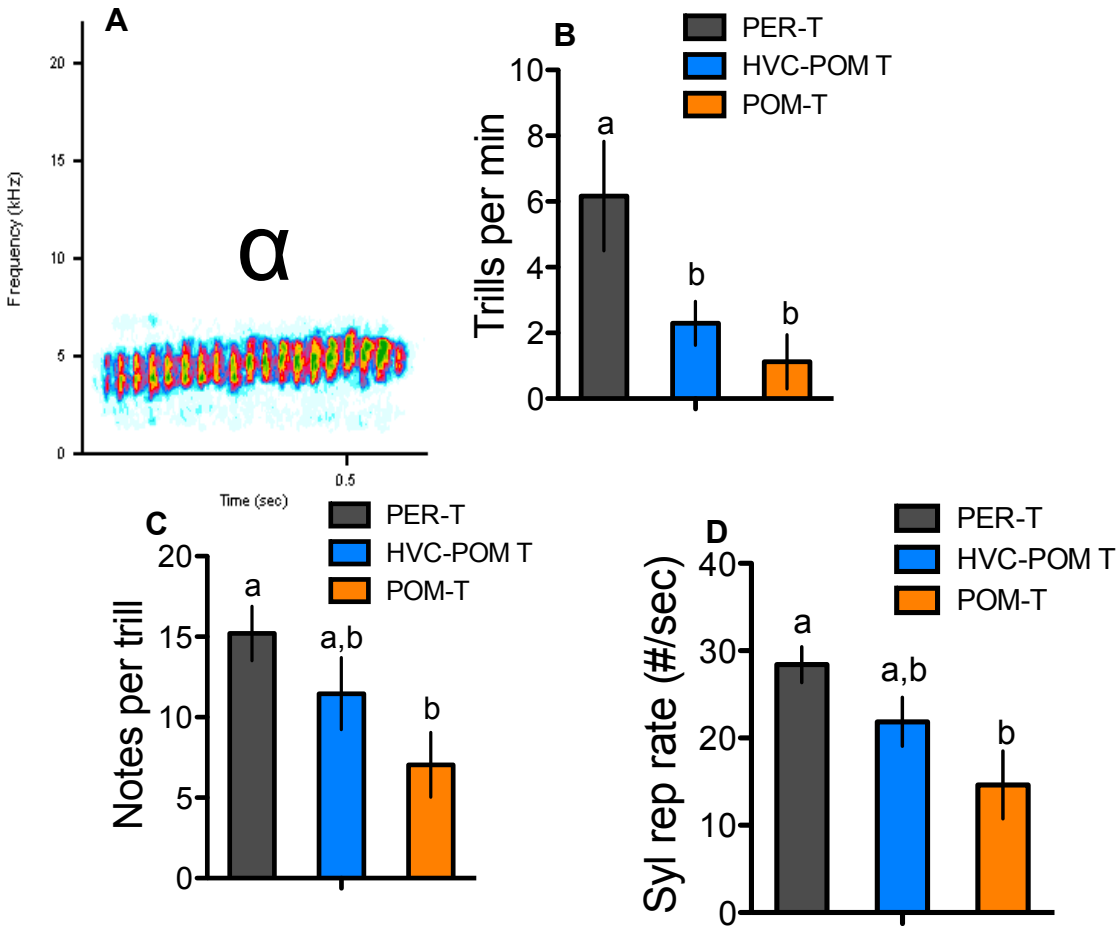

A
Frequency (kHz)
20
15
10
5
0
α
0.5
Time (sec)
B
Trills per min
10
8
6
4
2
0
a
b
b
PER-T
HVC-POM T
POM-T
C
Notes per trill
20
15
10
5
0
a
a,b
b
PER-T
HVC-POM T
POM-T
D
Syl rep rate (#/sec)
40
30
20
10
0
a
a,b
b
PER-T
HVC-POM T
POM-T

Figure 3

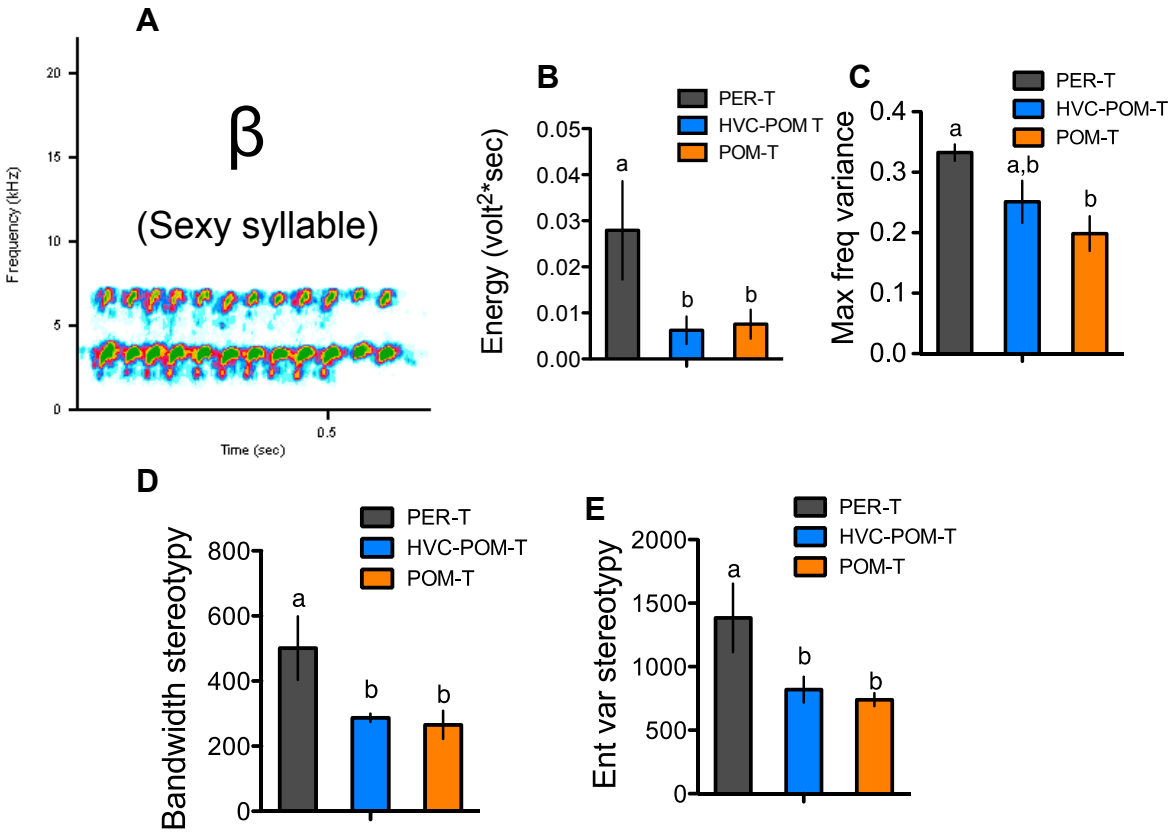
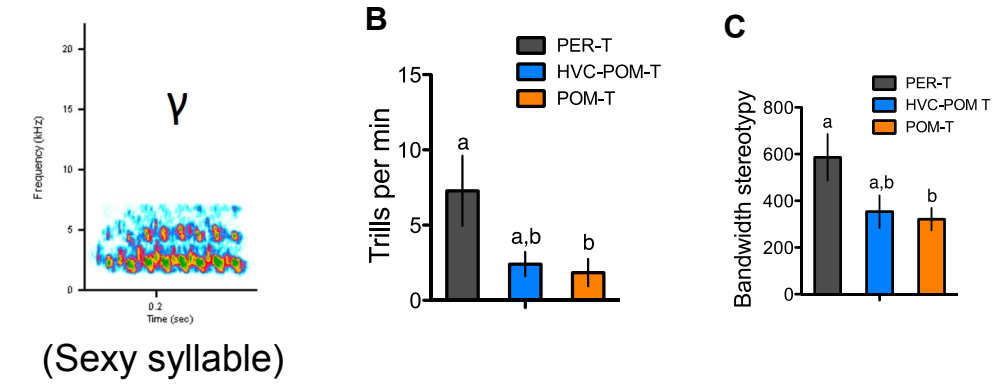

A
β
(Sexy syllable)
Frequency (kHz)
Time (sec)
B
Energy (volt²*sec)
PER-T
HVC-POM T
POM-T
a
b
b
C
Max freq variance
PER-T
HVC-POM-T
POM-T
a
a,b
b
D
Bandwidth stereotypy
PER-T
HVC-POM-T
POM-T
a
b
b
E
Ent var stereotypy
PER-T
HVC-POM-T
POM-T
a
b
b

Figure 4


A
γ
Frequency (kHz)
20
15
10
5
0
0.2
Time (sec)
(Sexy syllable)
B
PER-T
HVC-POM-T
POM-T
Trills per min
15
10
5
0
a
a,b
b
C
PER-T
HVC-POM T
POM-T
Bandwidth stereotypy
800
600
400
200
0
a
a,b
b

Chapter 7: Investigating the effects of flutamide, an androgen receptor antagonist, in HVC and RA on the song of canaries

Introduction

The previous experiments have provided strong support for the idea that T is acting at multiple regions in the songbird brain and periphery to coordinate different features of canary song into a single, functional response. In the previous two experiments, it was shown that T in the HVC of castrated male canaries that also had T in their POM increased the stereotypy of songs and partially song complexity. Moreover, T in the HVC plays a role in the regulation of sexually-relevant vocal signals within the canary song, trills, including sexy syllables. However, birds with T implanted in the HVC did not restore trills of a magnitude equivalent to intact birds, suggesting T actions in other regions are involved.

Multiple candidate sites exist for where T may act in the regulation of trills in addition to the HVC, such as the syrinx and RA. In chapter 3 I investigated the role of T in the periphery (presumably T at the syrinx specifically) in the regulation of multiple song features including trills using the androgen receptor antagonist BICAL, a compound that does not cross the blood-brain barrier (Furr and Tucker, 1996). RA is a candidate brain area because of its myotopic organization, which means that it directly regulates muscle action in the syrinx (Vicario, 1991). The current study aims to investigate the function of androgen action in RA as well as HVC by implanting flutamide, an androgen receptor antagonist (Baker et al., 1967), in these brain regions and assessing multiple features of canary song. The effects of implanting flutamide in the HVC of birds

with T circulating globally on song may be different from the effects of implanting T in the HVC of castrated birds, as other regions at which T acts probably play a role in regulating features distinct from stereotypy of whole songs.

Methods

Materials and Methods

Animals and pre-experimental manipulations. Canaries (*Serinus canaria*) of the Border strain were used for this study. Male and female canaries were obtained from a local breeder (Maryland Exotic Birds). Upon entry into the lab birds were placed on a short day (SD) photoperiod (8L:16D) for six weeks to induce photosensitivity (Nicholls and Storey, 1977; Dawson et al., 2001).

Experimental groups and stereotaxic implantation. Canaries were anesthetized using isoflurane gas and implanted with a 12-mm silastic implant filled with 10 mm of crystalline T in the same manner T implants were inserted in earlier chapters (Chapters 3, 4, 5). We implanted birds with supplemental T to increase the probability that all birds sang, given our observations in chapter 3 that 4 birds did not sing. Birds were then placed in a stereotaxic apparatus modified for use in small birds such as canaries with the beak holder placed 45° below the horizontal axis of the apparatus. We used the following stereotaxic coordinates to target the HVC: -.6 mm from the dorsal surface of the brain; anterior-posterior: -2.3 mm from the rostral tip of the cerebellum; and medial-lateral: ± 2.7 mm from midline. For RA, the following coordinates were used: -2.40 mm from the dorsal surface of the brain; anterior-posterior: -.9 mm from the

rostral tip of the cerebellum; and medial-lateral: ± 2.70 mm from midline. Each bird received bilateral implants aimed at HVC or RA using a Hamilton syringe fashioned to hold the 25-gauge cannula filled with flutamide or left empty. Cannula were lowered to the target coordinates and dental cement was applied around the implant. Excess portion of the cannula was clipped off after the cement had dried. The skin was then sutured over the implant and lidocaine and antibiotics were applied around the sutured portion of the skin using a Q-tip. Implants were made using blunted 25-gauge needles filled over a length of 2 mm with flutamide (Sigma Flutamide, F9397), based on the work of Alexandre and Balthazart (1986). Implants were cleaned using acetone and a Kimwipe to remove any antagonist that stuck to the outside of the tube. Once birds recovered, they were returned to individual, sound-attenuated chambers set to 14L:10D to simulate breeding photoperiods.

A total of 28 male canaries were used in this study. 15 canaries were implanted with cannula targeting their HVC: 12 birds were implanted with flut-filled cannula (HVCflut) and 3 birds were implanted with empty cannula (HVCnoflut). 13 canaries were implanted with cannula targeting their RA: 10 birds were implanted with flut-filled cannula (RAflut) and 3 birds were implanted with empty cannula (RAnoflut). The relatively lower numbers for the ‘noflut’ controls were chosen based on expected variation in implant sites and the fact that androgen receptor blockade in HVC and RA on song (for HVC) and spontaneous firing rate (for HVC and RA) is only effective when the implants are near the nucleus (i.e., within 1 mm from the nearest edge of the nucleus of interest; Meitzen et al., 2007). It was

further confirmed by statistical analyses if it is appropriate to place birds that received flut that was not near HVC or RA in the respective 'noflut' groups (see below).

Song recordings and analysis. Song recording and analysis settings (e.g., filter settings, sampling rate, etc) were identical to those in chapter 2. Each day video and audio recordings were made from 800h to 1030h (lights on at 800 h). The features listed in the Appendix will be quantified for whole songs on each day using the sound analysis program Avisoft and from these results determined song stereotypy (Alward et al., 2013; Meitzen et al., 2007). Based on work by Meitzen and colleagues (2007) and Alward and colleagues (2013), we analyzed song during an early (e.g., day 10), middle (e.g., day 14), and later time point (e.g., day 21, the last day of treatment). However, we found that on the early and middle days sometimes certain birds did not sing during our recording time. Therefore, we used time blocks over days 10-12 and days 13-14, which allowed us to include all birds in our analyses. This allowed for repeated-measures ANOVAs and thus increased our statistical power.

Brain and blood collection and verification of implants. Twenty-one days after treatment initiation, birds were deeply anesthetized (4% Isoflurane), weighed, and their brain (which was also weighed) was extracted and fixed in acrolein after collecting blood from the trunk region into 1.5 ml centrifuge tubes. Blood was spun down at 8,000 rpm for 6 minutes and serum was collected and placed at -20° C. Brains were agitated in acrolein for 2 hours, then washed for 15 minutes four times in phosphate buffered saline (PBS) and placed in sucrose

(30% solution in PBS) over night until they sank to the bottom of the vial. After cryoprotection by sucrose, brains were flash frozen in dry ice for 5 minutes, and then placed into a -70° C freezer. At autopsy, the presence of the systemic T implant as well as the state of the testis was confirmed.

Brain analyses. Brains were sectioned using a cryostat at 30 microns into four series of sections that were stored in cryoprotectant. These four series were placed into a -20° C freezer. One series was later mounted on gelatin-coated slides and exposed to air for a day. Then, mounted sections were exposed to a standard Nissl staining procedure and coverslipped using Permount (Fisher Scientific). Based on these stained sections, the positions of the implant centers were drawn onto a series of modified atlas plates obtained from the canary atlas made by Stokes et al (1976) using the revised nomenclature for the songbird brain (Reiner et al., 2004)

Statistical Analyses. Mixed-Design ANOVAs (Treatment*Day) were used to assess the effects of flutamide treatment. If the requirements for conducting parametric statistics were not met, separate non-parametric repeated-measures ANOVAs were run on the individual treatment groups (there currently does not exist a method for conducting a non-parametric counterpart to two-way repeated measures ANOVAs). Following significant interaction components in the omnibus parametric ANOVAs, Scheffe's contrasts were used to make comparisons based on effects. When non-parametric omnibus ANOVAs yielded a significant effect,

Wilcoxin's tests or Mann-Whitney post hoc tests were used. Effects were concluded to be significant at $p \leq 0.05$ using two-tailed tests.

In rare instances, there were no effects of treatment or an interaction but when the data were visualized it appeared differences may exist on one or two days but these differences may have been washed out due to non-differences on one or two other days. In all of these instances the effect of treatment had p values between 0.1 and 0.2. Therefore, to avoid committing type-II errors, t-tests were conducted on days where it appeared differences might exist. We used an alpha level of 0.02 for these comparisons (per Sidak correction for two comparisons). The number of comparisons never exceeded two.

Results

Verification of implant sites and treatment groups. Some birds had lesioned HVC (3 birds) or lesioned RA (1 bird) or only received a unilateral implant near HVC (1 bird) or RA (2 birds) instead of bilaterally. Due to the sparse nature of this variation, these birds could not be placed into a specific treatment group and were thus excluded from statistical analysis.

A total of nine birds received flut bilaterally near their HVC (in every instance cannula tips were directly lateral to HVC). After determining that birds that received bilateral flut implants not near HVC (n=2) were no different in terms of all song features compared to the HVCnoflut group, these birds were placed in the HVCnoflut group. Overall, the final treatment groups for HVC were as follows: HVCnoflut, n=4 and HVCflut, n=9.

A total of four birds received flut bilaterally near their RA (canula tips were usually laterally and slightly ventral to RA). Three birds were found to have bilateral flut implants outside of RA and, for the same reasons as above for HVC, these birds were placed in the RAnoflut group. Thus, the final treatment groups for RA were: RAnoflut, n=5 and RAflut, n=4.

Androgens in HVC and RA are not involved in regulating the motivational measures of song. There was no effect of treatment on song rate for birds implanted near HVC or birds implanted near RA ($p \geq 0.37$ for both ANOVAs; graph not shown) or an interaction between treatment and day ($p \geq 0.12$ for both ANOVAs). There was a significant effect of RA treatment on song duration (treatment, $p<0.05$; treatment*day, $p=0.36$; day, $p=0.64$; graph not shown). This difference was not parsed out using Scheffe's contrasts; however, this is not surprising given the p value for treatment ($p=0.047$). There was no effect of HVC treatment on song duration (treatment, $p=0.15$; treatment*day, $p=0.18$; day, $p=0.45$; graph not shown).

Androgens in HVC and RA play overlapping yet dissociable roles in regulating songs stereotypy and acoustic structure. Blocking AR in either HVC or RA caused changes to multiple song features (Figure 1). For instance, blocking AR in HVC and RA reduced the stereotypy of song. Flutamide in HVC caused birds to sing with less bandwidth stereotypy on day 21 compared to the two earlier time points (for bandwidth stereotypy, non-parametric omnibus ANOVA for HVC-flut, $p<0.05$; days 10-12 and 13-14 versus day 21, $p<0.05$ for both post-hoc analyses) and with less entropy variance stereotypy on day 10-12

(between-subjects t-test, $p<0.02$). Flutamide in RA also caused birds to sing with less bandwidth stereotypy compared to untreated birds and this effect occurred quickly and only manifested as a between-subjects effect (parametric omnibus ANOVA effect of treatment for both stereotypy variables, $p<0.05$; for bandwidth stereotypy, RAflut versus RAnoflut, days 10-12 and days13-14, $p<0.05$ for both comparisons; for entropy variance stereotypy, $p<0.05$ for day 21).

Birds treated with flutamide in RA sang songs that were less complex in terms of bandwidth variance compared to untreated birds (omnibus parametric ANOVA, $p<0.05$; within-subjects contrasts day 10-12 and 13-14 versus day 21, $p<0.05$ for RAflut). This was not observed in birds treated with HVCflut versus HVCnoflut (parametric ANOVA, effect of treatment, $p=0.92$; treatment*day, $p=0.69$). Moreover, on day 13-14, RAflut birds tended to sing songs that were higher in energy compared to RAnoflut birds (t-test, $p=0.09$).

Androgens in RA regulate trill acoustic structure and stereotypy more robustly and differentially compared to androgens in HVC. There was interaction for birds treated with HVCflut versus HVCnoflut (omnibus parametric ANOVA, treatment*day, $p<0.05$) such that from days 10-12 and days 13-14 to day 21, HVCflut birds decreased their trill bandwidth stereotypy while HVCnoflut birds increased their trill bandwidth stereotypy (Figure 2). There was no effect for HVC treatment on the entropy variance stereotypy of trills. There was a main effect of RA treatment for the two measures of trill stereotypy (omnibus parametric ANOVAs, treatment effect, $p<0.05$ for both). For instance, on day 10-12 RAflut birds sang trills with less bandwidth stereotypy than RAnoflut birds

($p<0.05$). On days 13-14 and day 21, RAflut birds tended to sing trills with less bandwidth stereotypy compared to RAnoflut birds ($p=0.09$ and $p=0.06$, respectively). A similar pattern was observed for entropy variance stereotypy, where RAflut birds sang trills with less stereotypy on days 10-12 and 13-14 ($p<0.05$ for both) and tended to sing with less stereotypy on day 21 ($p=0.09$).

RAflut birds sang trills with less bandwidth variance compared to RAnoflut birds (omnibus parametric ANOVA effect of treatment $p<0.05$), such that on days 10-12 and 13-14, RAflut birds sang trills with less bandwidth variance compared to RAnoflut birds ($p<0.05$ for both comparisons). There was no effect of HVC treatment on bandwidth variance. However, there was a main effect of HVC treatment on trill duration (omnibus parametric ANOVA effect of treatment, $p<0.05$), in which HVCflut birds sang longer trills on days 10-12 and 13-14 ($p<0.05$ for both comparisons). There was no effect of RA treatment on trill duration (ANOVA, treatment, $p=0.83$; interaction, treatment*day, $p=0.89$; day, $p=0.31$). For both HVC and RA, there was no effect of treatment (parametric ANOVA, $p\geq0.44$) or treatment*day, $p\geq0.55$). For HVC, there was a main effect of day ($p<0.05$), wherein trill repetition rate increased over time (linear contrast, $p<0.05$). This was not observed for RA (effect of day, $p=0.36$); however, there tended to be a quadratic relationship between treatment and day ($p=0.09$).

Discussion

Birdsong is differentially regulated by androgens in HVC versus RA. In line with the work presented previously (i.e., chapters 4 and 5) as well as the work of

others (Meitzen et al. 2007), androgens acting within HVC do not play a role in regulating the motivation to sing. Based on the results here, that same principle can be applied to androgens in RA. However, we have shown here that androgens in HVC and RA are critical to regulating the acoustic structure and stereotypy of songs and trills. This regulation appears to be dissociable based on androgens acting in HVC versus RA, with some features being regulated to differing degrees or completely different features being regulated by androgens in HVC or RA.

Androgens in HVC and RA play overlapping as well as differential song regulatory roles. The results of this study show that both HVC and RA are important sites for androgens to regulate the acoustic structure and stereotypy of song. For instance, androgens acting in HVC and RA are required to maintain song stereotypy. Blocking androgens in RA, however, led to a much larger decrease in song stereotypy compared to HVC and this decrease happened a lot sooner. One explanation could relate to proximity of the brain region to the effector organ—in this instance, the effector organ is the syrinx and since RA is more closely linked to controlling the fine-motor output of the syrinx (in space and its myotopic organization), blocking AR in RA may lead to a more substantial effect on song stereotypy (Vicario 1991). What follows from this interpretation is that in terms of androgenic action in HVC and RA, RA is not solely relaying exactly what HVC activity is instructing it to do, and something about RA's intrinsic activity, presumably being driven by AR activation, controls song stereotypy; this would be I line with Spiro and colleagues (1999), showing that

spontaneous bursts in RA can modulate afferent input from HVC and LMAN. Another likely explanation of where AR are important in RA for regulating song features are the LMAN-RA synapses (White et al., 1999). Indeed, it is possible that we blocked AR where both HVC-RA and LMAN-RA synapses are present. Blocking AR in RA caused perturbations in both stereotypy and complexity while blocking AR in HVC only caused perturbations in stereotypy. As LMAN is thought to inject variability into RA via NMDA-modulated excitatory-post-synaptic potentials (EPSPs), which are modulated by androgens (White et al., 1999), we may have affected those synapses here.

It would be premature, however, to state that the actions of androgens in HVC do not play a more critical role in regulating song acoustic structure and stereotypy. Indeed, in chapter 5, we showed that T in the HVC and POM was able to fully restore song stereotypy. Moreover, Meitzen and colleagues (2007) have shown that both AR and ER in HVC contribute to the regulation of song stereotypy and maintain spontaneous firing rate in RA. This would imply that T and its metabolites, such as estradiol, work in combination to regulate song stereotypy. Therefore, in the current study it may be that activation of ER in HVC were able to maintain, at least for a short time, song stereotypy. Combined with the observation that AR blockade in RA caused a substantial reduction in song stereotypy, this would suggest that even though AR and ER in HVC maintain song stereotypy, AR in RA are permissive for this regulatory mechanism to occur (Meitzen et al., 2007).

Interestingly, we observed blocking AR in HVC caused an increase in trill duration compared to untreated birds. We also saw that birds treated with flutamide in RA tended to produce louder songs compared to untreated birds. When these effects are viewed in combination, they may be at least partially explained based on work conducted by Long and Fee (2008), wherein they altered temperature in HVC and RA to show that HVC is involved in the precise timing of the duration of vocalizations while RA is involved in the specific acoustic structure of vocalizations. These results are also in line with work by Sober et al. (2008), showing that RA activity is tightly linked to song amplitude. This suggests that AR activation in HVC and RA may partially be involved in regulating these differential effects) Bottjer and Hewer 1992; White et al. 1999).

However, as mentioned in chapter 3, we have hypothesized that the observed increases in energy and duration may be due to the bird being a state of sensorimotor vocal variation caused by perturbed auditory feedback. Indeed, increases in the duration and amplitude of vocalizations are two key observations made in an animal that has experienced perturbed auditory feedback and is thought to be in a state of trying to match its auditory template. Indeed, Cynx and Rad (2001) showed that zebra finches experiencing delayed auditory feedback increase the amplitude of their songs. This hypothesis warrants further investigation.

Androgens in HVC may transsynaptically influence RA to regulate song stereotypy and acoustic structure. In chapter 5 we showed that T in the HVC and POM of canaries was sufficient to fully restore song stereotypy and was able

to partially restore complexity measures. In light of the observations made here, that blocking androgen receptors in RA substantially reduced song stereotypy and complexity, it is likely that T in the HVC can transsynaptically influence RA to regulate song. This is concordant with the results from Meitzen and colleagues (2007) showing that blocking AR in HVC decreased spontaneous firing rates in RA and AR in RA can cause the same decrease in firing rate in RA. However, this transsynaptic regulation by AR appears to be subtle, as blocking AR in HVC caused less robust changes in song features as did RA, indicating that AR in RA possesses the intrinsic ability (Spiro et al., 1999) to regulate these song features or both AR and ER must be activated for this transsynaptic regulation to occur (see above and Meitzen et al., 2007). It also may be the case that other synapses, such as between LMAN and RA, are involved in regulating some of these acoustic features (Bottjer and Hewer 1992; White et al. 1999) and we affected those sites in the present study. In support of this prediction, LMAN is postulated to generate vocal variability via RA (Brainard and Doupe, 2000b; Kao et al., 2006; Rouse and Ball, 2015; Sober et al., 2008), and in this study measures of within-song variability were perturbed in RAflut birds. Thus, the role of androgens in LMAN and androgens specifically at LMAN-RA synapses in the regulation of birdsong warrants further investigation.

Regulation of song by AR in HVC versus RA is not as simple as a large versus small song unit dichotomy. Based on the hypothesis put forward by Margoliash (1997), and our observations made in chapter 6, we predicted that blocking AR in HVC would cause perturbations in song acoustic structure and

stereotypy via disrupting overall ‘larger’ features of songs (e.g., whole songs) while blocking AR in RA would also cause perturbations in song, but via disrupting smaller units of songs (e.g., trills). While it may be the case that HVC and RA do indeed encode for differently-sized units of song, activation of AR in these nuclei does not appear to modulate such a representation to a substantial degree. For instance, although we saw blockade of AR in RA cause a large decrease in entropy variance stereotypy of trills and the same was not observed for HVC, blockade AR in HVC caused a decrease (albeit smaller) in bandwidth stereotypy of trills as did AR in RA. For song, activation of AR in both RA and HVC were required for maintaining stereotypy. Hence, while there is some evidence AR in RA plays a specific regulatory role in trill stereotypy, AR in both nuclei are involved in regulating both trills and song. This would indicate that our results in chapter 6, that T in HVC and POM only partially restored the acoustic structure of trills, is because T in RA was required *as well*, not instead. Hence, perhaps it is the case that as the units of song get progressively smaller, the role of AR in both nuclei becomes more pronounced. Nonetheless, it is possible a clearer distinction may be present when looking at even smaller units of song, such as individual notes are syllables (Spiro et al., 1999).

Conclusion. Birdsong is a complex learned behavior with multiple levels of organization. As studies on the various levels at which T can act in the regulation of birdsong are performed, it has become apparent that T is acting in multiple levels of the brain and periphery to regulate features of song such as the

motivation to sing, song stereotypy, song complexity, and all of these features with respect to the different units within songs (e.g., trills and other vocalizations). The results of the current study help to elucidate our understanding of how steroid hormones such as T coordinate all of these features into a single adaptive response. Questions still remain in terms of the critical sites of steroid hormone action in the regulation of birdsong, such as the role of AR in LMAN and the role of estrogens in these processes.

Figure legend

Figure 1- Effects of treatment on song. The left column shows the effects for treatment of HVC with flutamide or an empty cannula. The right column shows the effects for treatment of RA with flutamide or an empty cannula. Each point over each day corresponds to mean values and error bars are SEM. # denotes a significant effect of treatment. An asterisk indicates a significant effect over time. For bandwidth stereotypy for HVC, the asterisk denotes a significant effect of day within the HVCflut group but not HVCnoflut. For bandwidth variance, the asterisk denotes a significant effect of day within the RAflut group but not RAnoflut. Differences were considered significant at $p \leq 0.05$. For HVC, # denotes a significant difference at an alpha level of 0.02.

Figure 2- Effects of treatment on trills. The left column shows the effects for treatment of HVC with flutamide or an empty cannula. The right column shows the effects for treatment of RA with flutamide or an empty cannula. Each point over each day corresponds to mean values and error bars are SEM. # denotes a significant effect of treatment. An asterisk indicates a significant effect over time. For bandwidth stereotypy for HVC, the asterisk denotes a significant treatment*day effect, specifically a crossover interaction. Differences were considered significant at $p \leq 0.05$.

Figure 1

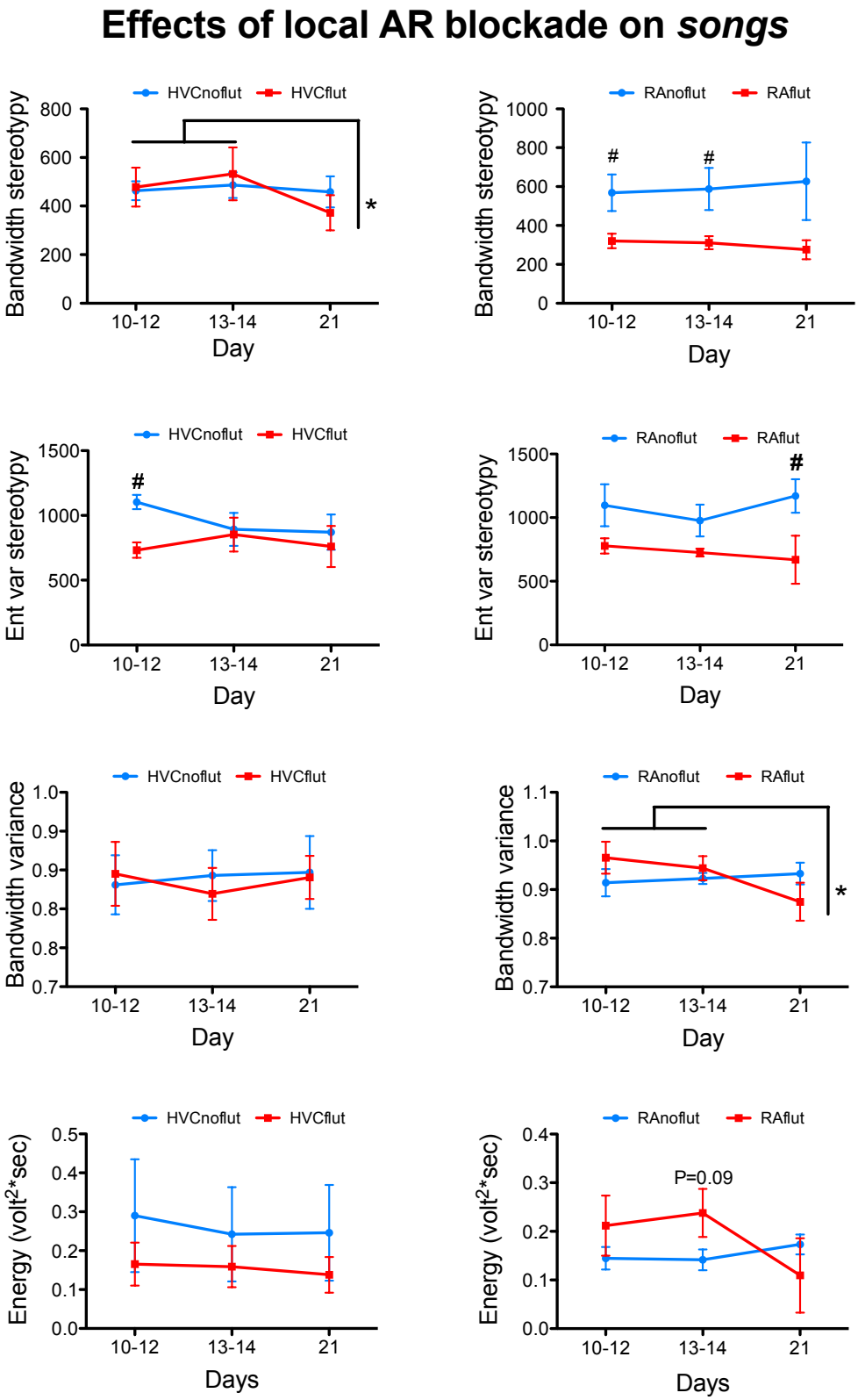
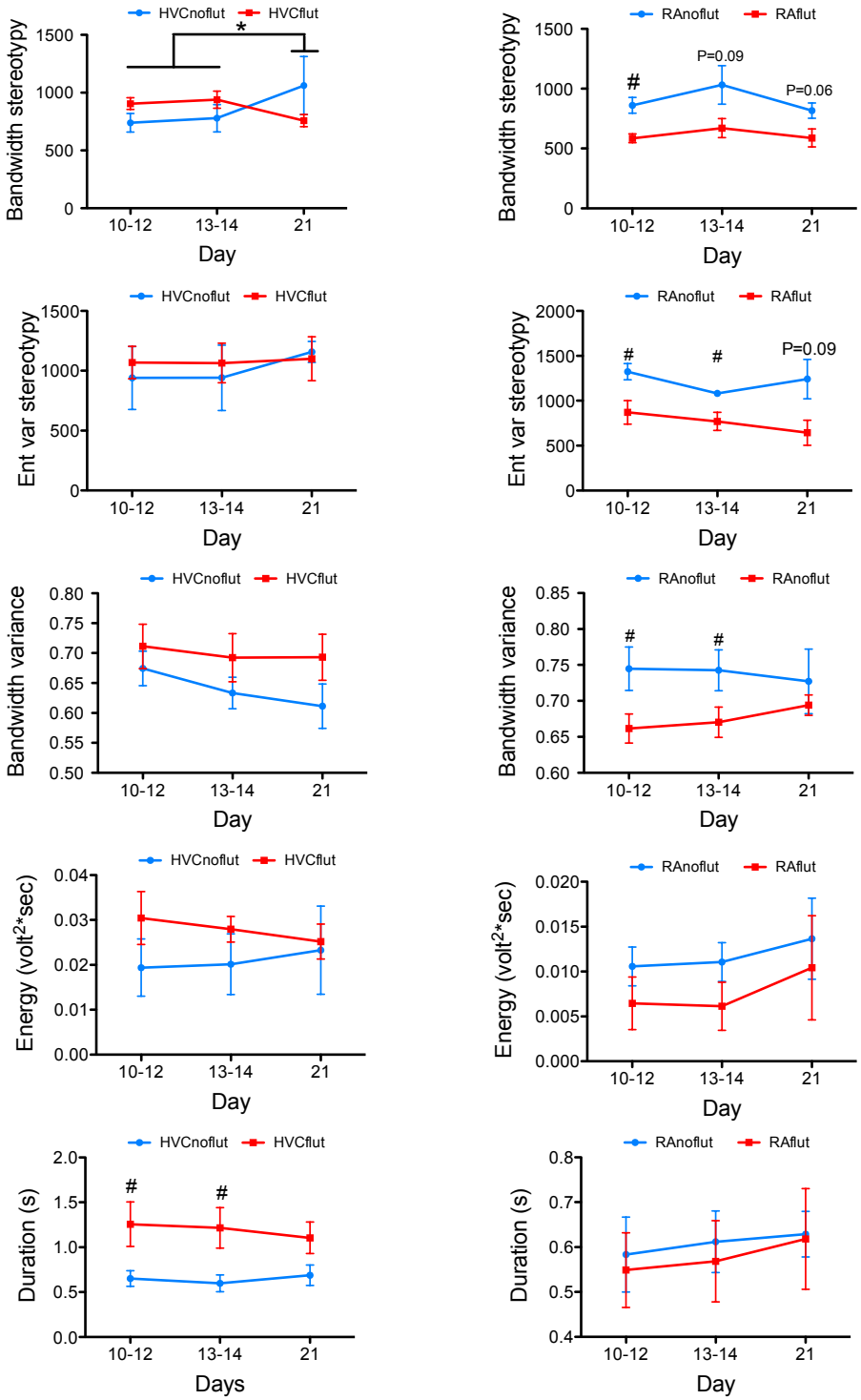

Effects of local AR blockade on *songs*
HVCnoflut
HVCflut
RAnoflut
RAflut
Bandwidth stereotypy
Ent var stereotypy
Bandwidth variance
Energy (volt²*sec)
Day
Days
10-12
13-14
21
#
*
P=0.09

Figure 2


Effects of local AR blockade on *trills*
HVCnoflut
HVCflut
RAnoflut
RAflut
Bandwidth stereotypy
Ent var stereotypy
Bandwidth variance
Energy (volt²*sec)
Duration (s)
Day
Days
10-12
13-14
21
*
#
P=0.09
P=0.06

Chapter 8: Evidence for fast, non-genomic-like actions of estrogens in the regulation of canary song

Introduction

The previous studies in this dissertation have focused primarily on the genomic actions of T, assessing behavioral changes over periods of a few to several days (Adkins-Regan, 2009; Ball et al., 2002). A plethora of recent evidence, however, has shown that steroid hormones, such as E2, can also have rapid, short-term effects on behavior by acting in a non-genomic manner (Cornil, Ball, and Balthazart, 2006; Cornil et al., 2013, 2012; Remage-Healey, et al., 2010; Remage-Healey, 2012; Seredynski, et al., 2013). Song behavior is well known to be regulated by steroid hormones such as T. Castrated canaries (*Serinus canaria*) substantially reduce song output and treatment with exogenous T restores singing after approximately three days (Alward et al., 2013; Sartor et al., 2005). Moreover, in zebra finches (*Taeniopygia guttata*) treatment with an inhibitor of aromatase, the enzyme that converts T into estradiol, reduces song output (Walters, Collado, and Harding 1991; Walters and Harding, 1988). Interestingly, recent studies in male Japanese quail indicate that brain-derived estradiol acts on a fast time scale (within minutes) to regulate sexual behavior and these effects dissipate within 2 hours, suggesting these effects are non-genomic (Seredynski et al., 2013). These effects are also mimicked by membrane impermeable estrogens, further indicating a non-genomic mechanism (Seredynski et al., 2013).

Throughout the songbird brain there are multiple potential sites of steroid action (Ball et al., 2002; Bernard et al., 1999). For instance, androgen receptors are expressed in HVC, RA, lMAN, and throughout the hypothalamus and midbrain and ERα are expressed in HVC in some songbird species as well as in the hypothalamus. Aromatase is also widely distributed throughout the songbird brain and its distribution is largely in line with what is present in rodent species (Naftolin, Horvath, and Balthazart 2001) although its activity is much higher in the songbird brain (Forlano et al., 2006). For instance, aromatase is distributed throughout the hypothalamus, including areas like the preoptic area (POA) including the medial preoptic nucleus (POM) (Balthazart et al., 1996) and the telencephalon (Balthazart, et al., 1996; Saldanha et al., 2000; Shen et al., 1995). Especially important for the present investigation, aromatase is noticeably absent from song control regions such as HVC and RA but very densely expressed in the auditory regions NCM and CMM. Thus, if the conversion of T to estrogens via aromatase is involved in the rapid activation of birdsong it could occur at multiple levels of the brain while regulating different aspects of this complex behavior. Here, we investigated the fast-acting, presumably non-genomic influence of estrogens in the regulation of song in male canaries by examining the effects of acute aromatase inhibition on the motivation to sing and song acoustic structure and stereotypy.

Methods

Materials and Methods

Animals and pre-experimental manipulations. Canaries (*Serinus canaria*) of the American singer strain were used for this study. Male and female canaries were obtained from a local breeder (Maryland Exotic Birds). Upon entry into the lab birds were placed on a short day (SD) photoperiod (8L:16D) for six weeks to induce photosensitivity (Dawson et al., 2001).

Experimental manipulations (effects of fadrozole). A total of 12 male canaries were used in this study. Each bird was placed in individual, sound attenuated chambers on long days (14L:10D) to simulate breeding photoperiods. After 10 days of acclimation, 5 minutes following lights on, birds were either injected with fadrozole (30 mg/kg), a potent, non-steroidal, reversible aromatase inhibitor, with propylene glycol (propylene glycol:saline=4:1) as the vehicle or vehicle only (Cornil, et al., 2006). Injections were made intraperitoneally by using forceps to lift up the skin beneath the keel and inserting the needle and immediately making the injection. Another person would grab the bird from its cage and hold it in their hand while another person made the injection. The whole injection procedure took less than a minute in almost all cases. Treatments were made using a within subject design, with three days between each injection. After birds' second injection, three days later they were injected with either vehicle or fadrozole and 30 minutes later their brains were rapidly extracted and frozen on dry ice. These

brains were to be used to assess the efficacy of fadrozole on aromatase inhibition (see below).

Experimental manipulations (effects of fadrozole + estradiol). A total of 17 male canaries were used for this study. Acclimation procedures were identical to the above methods. Between two and five minutes after lights on following the acclimation period, birds were either injected with vehicle, fadrozole, or fadrozole plus estradiol (2.5 mg/kg). Crystalline estradiol was mixed with the fadrozole plus vehicle mixture, and, as has been observed in previous work (Cornil et al 2006) it did not go into solution without several drops of 100% ethanol to increase solubility. Injections were made in an identical fashion as above. Instead of using a three-day gap period between injections as above, we waited four days between each injection, given that we were giving more injections and to minimize possible carry-over effects of negative effects of estradiol or stress due to multiple injections. After the third injection, we gave a final injection of either fadrozole or vehicle and brains were extracted either 30 minutes or 4 hours later. The latter time point was chosen based on observations from the first experimental manipulation that most birds treated with fadrozole begin showing a rebound in song behavior by about 4 hours, suggesting aromatase activity has returned to normal levels. This is in line with rebound effects observed by Cornil and colleagues (2006) in male Japanese quail injected with fadrozole intraperitoneally.

Microdissections and measuring aromatase activity. To assess the efficacy of fadrozole in inhibiting the activity of aromatase, we microdissected two regions of the brain that are well known to express very high levels of aromatase, the hypothalamic-preoptic area (HPOA) and the NCM and ran an assay measuring aromatase activity (AA). The method used for microdissecting out the HPOA was modified from that used by Cornil and colleagues (2006b) in quail for use in canaries (See Figure 2). Briefly, microdissections were made in the HPOA from the presence of the supraoptic decussation as it spans across the midline until the anterior commissure just begins to no longer span the midline. This resulted in the collection of three microdissected HPOA from all birds. For NCM, an area flanking the midline was microdissected out from where the anterior commissure crossed the midline and intersected with the occipitomesencephalicus to the posterior commissure, leading to the collection of five microdissected sections from all birds. To ensure aromatase from the hippocampus did not confound the activity present in the NCM region that was microdissected, the hippocampus was removed using a razor blade.

The aromatase activity assay was performed almost identically to that used by Cornil et al (2006a) with only minor modifications. The microdissected regions were homogenized with a glass homogenizer in ice-cold buffer containing 150 mM KCL, 1 mM Na-EDTA, 10 mM Tris-HVC pH 7.2. Aromatase activity was quantified by measuring the tritiated water production from [1β-^{3}H]-androstenedione (Roselli and Resko, 1991). On an ice bath, triplicate aliquots (50 µl) of homogenate containing approximately 1 mg wet weight were added to

50 µl of 100 nM [1β-^{3}H]-androstenedione and 50 µl of buffer. To initiate the assay, 50 µl of NADPH was added so as to reach a final concentration of 1.2 mM. All these steps were conducted at 4°C in 1.5-ml Eppendorf tubes which were then quickly capped and incubated for 20 min (as opposed to 15 min for Japanese quail) at 37°C. The reaction was stopped by cooling the sample in an ice bath and adding 0.4 ml ice-cold 10% trichloroacetic acid containing 2% activated charcoal. After centrifugation at 1200 *g* for 15 min, supernatants were applied to small columns made of Pasteur pipettes plugged with glass beads and filled (3 cm high) with a Dowex cation exchange resin AG 50W-X4, 100–200 mesh (Biorad, Richmond, CA). The columns were then eluted with 3 × 0.6 ml distilled water. Effluents were collected in scintillation vials and 10 ml Ecoscint A (National Diagnostics, Atlanta, GA) were finally added. Vials were counted for 3 min on a Packard Tri-Carb 1600 TR Liquid Scintillation analyzer.

Within each experiment, blanks were obtained by processing brain samples in the presence of an excess (final concentration about 40 µM) of the potent and specific aromatase inhibitor, R76713 (Racemic vorozole, Janssen Pharmaceutica, Beerse, Belgium). The blank values never exceeded 140 dpm while active control samples had radioactivities ranging between 2000 and 3000 dpm. A recovery of 93 ± 2% was usually obtained from samples of 10,000 dpm tritiated water conducted throughout the entire purification procedure (incubation, centrifugation and Dowex column). Enzyme activity was expressed in fmol h^{-1} after correction of the counts for quenching, recovery, blank values and percentage of tritium in β-position in the substrate.

Song recordings and analysis. Song recording and analysis settings (e.g., filter settings, sampling rate, etc) were identical to those in chapter 2. On injection days, song was recorded from lights on (800 h) to 1800 h. This allowed for analysis of changes in song over time after injection. On the day before the first injection song was recorded from 800 h to 1030 h to provide a baseline level of singing. Then, on the day following each injection song was recorded for 800 h to 1030 h. Song was also recorded the day before each injection to allow for analysis of general changes in song behavior over time (e.g. changes cause by repeated injections). These various recording procedures allowed for answering two important research questions. Being able to assess song the day after injections and comparing them to the baseline recording, one can ask if the effects of aromatase inhibition are long-lasting. If the effects occur within the same day of injection of fadrozole, and are gone by the day after treatment, it can be assumed, albeit to a small degree, that song may be regulated in a non-genomic fashion by the activity of aromatase. Motivational measures, such as the latency to sing, % time spent singing, and song duration were measured. The appendix lists the song acoustic features that were assessed and the stereotypy of these features was computed.

Brain and blood collection. Thirty minutes or four hours (see above) after birds received their last injection, they were removed from their cage and their brains were extracted and immediately placed on dry ice and blood collected from the trunk region into 1.5 ml centrifuge tubes. Brains were also weighed after being in

dry ice for at least five minutes by placing them in aluminum foil on a scale and immediately placing in a -70° C fridge.

Statistical Analyses. Within-subjects statistical analyses were used to assess the effects of treatment when appropriate. Effects were considered significant at $p \leq 0.05$ using two-tailed statistical analyses. Based on the results of the first experiment, we had relatively strong a priori directional hypotheses for experiment II. Hence, in some cases where we had directional hypotheses, we showed the results of both two-tailed t-test and one-tailed.

Results I: Fadrozole injection experiment

Fadrozole reduced aromatase activity within 30 minutes, but aromatase activity had rebounded four hours later. For the HPOA, there was a marginal effect of treatment on aromatase activity and latency of brain extraction ($P=0.09$ and 0.08, respectively). However, there was a significant interaction between these two variables ($P<0.05$). Indeed, 30 minutes after fadrozole treatment led to a substantial reduction in aromatase activity compared to vehicle ($P<0.05$); however, this difference had disappeared four hours after injection. For the NCM, there was a significant effect of treatment and latency of brain extraction ($P<0.05$) but no interaction. Importantly, however, 30 minutes after injection fadrozole caused a large reduction in aromatase activity relative to vehicle ($P<0.05$), a difference that was no longer present at four hours after injection.

Acute aromatase inhibition leads to a decrease in the motivational measures of song. On the day of injections, birds treated with fadrozole sang

significantly later following injection than birds treated with vehicle ($P<0.05$; Figure 4). Fadrozole-treated birds also spent less time singing and sang shorter songs ($p<0.05$ for both).

Acute aromatase inhibition perturbs the acoustical parameters of song. Fadrozole treatment caused perturbations in the acoustic features of song (Figure 5). Birds treated with fadrozole sang songs with lower energy compared to birds treated with vehicle. These birds also sang with a lower minimum frequency and a lower fundamental frequency ($P<0.05$ for all comparisons).

Acute aromatase inhibition reduces the stereotypy of song. Fadrozole treatment also caused a decrease in the stereotypy of song (Figure 5). Birds treated with fadrozole sang songs with lower bandwidth stereotypy as well as lower entropy stereotypy ($P<0.05$ for both comparisons).

The effects of acute aromatase inhibition are short-lasting, but some attributes of song are perturbed the day after. Every single feature of song that was perturbed on the day of fadrozole treatment was back to normal levels the day after treatment ($P \geq 0.26$ for all comparisons). Interestingly though, entropy was reduced in fadrozole-treated birds relative to vehicle-treated birds ($P<0.05$).

Results II: Effects of fadrozole+estradiol and time-course analysis

Three birds did not sing on any of the injection days while 1 bird only sang on one injection day—since this birds could not be included in a within-subjects analysis they were thus excluded from the analysis. To conduct a time-course

analysis on the effects of fadrozole and fadrozole plus estradiol on song, we broke the song analysis into two-hour time bins starting at 0800h and going until 1800h. When analyzing the effects of treatment on the motivational measures of song, it became apparent that the zero values of % time spent singing and song rate were preventing a reliable or meaningful statistical analysis of these measures. Because the reasons a bird may not sing throughout the whole day could be difficult to elucidate (e.g., due to negative reactions to injections) and the many zero values at certain time points which will prevent a reliable statistical analysis, for the two motivational measures we focused our statistical analyses on only birds that sang on each day they were injected (n=5).

Moreover, given that every bird did not sing on each day or at individual time-points and that we cannot use zero values for any of the duration, acoustic, or stereotypy measures of song, we were not able to conduct within-subjects ANOVAs. Therefore, we performed paired t-tests when a comparison between the effects of injections was available at the individual time points. For instance, if a bird sang when it was injected with vehicle between 1200 and 1400 and fadrozole plus estradiol between 1200 and 1400, but not when it was injected with fadrozole between 1200-1400, we conducted a paired t-test for the effects of vehicle versus fadrozole plus estradiol. Along the same lines, the only meaningful comparisons that were able to be made in the morning were if we collapsed the time points in the morning into 0800-1200, as opposed to 0800 to 1000 and 1000 to 1200, as was possible for the motivational measures of song. All of the later time points were the same. When these data were graphed all birds that sang at

each time point, however, were included to allow for visualization of the overall mean values on each injection day. We were able to make the following comparisons for duration, stereotypy, and the acoustic features:

- 0800-1200: VEH versus FAD, n=4; VEH versus FADe2, n=3; FAD versus FADe2, n=3
- 1200-1400: VEH versus FAD, n=6; VEH versus FADe2, n=3; FAD versus FADe2, n=3
- 1400-1600: VEH versus FAD, n=7; VEH versus FADe2, n=5; FAD versus FADe2, n=5
- 1600-1800: VEH versus FAD, n=7; VEH versus FADe2, n=8; FAD versus FADe2, n=6

Estradiol depletion caused by fadrozole leads to rapid, short-lasting perturbations in most song features. When treated with fadrozole, birds sang fewer songs between 0800 and 1000 compared to when they were injected with vehicle (Figure 7; within-subjects t-test, $p<0.05$) but these differences disappeared for the rest of the day (all within-subjects comparisons, $p \geq 0.39$). A similar pattern was observed for % time-spent singing, with fadrozole causing a reduction in the amount of time birds spent singing between 1000 and 1200 compared to vehicle (within-subjects t-test, $p=0.06$; 1-tailed, $p<0.05$) but these differences disappeared for the rest of the day (all within-subjects comparisons, $p \geq 0.34$). There was also an effect of treatment on the duration of song, with fadrozole causing a reduction in the duration of songs in the early afternoon (between 1200 and 1400 within-subjects t-test, $p=0.06$; 1-tailed t-test, $p<0.05$; 1400-1600, within-subjects t-test, $p<0.05$) but this difference disappeared in the evening (1600-1800, within-subjects t-test, $p=0.13$). When birds were treated with fadrozole plus estradiol, no perturbations were observed for any of the above

motivational measures relative to when they were treated with vehicle or fadrozole (within-subjects t-test, $p \geq 0.23$ for all comparisons.

Treatment also caused disruptions in the acoustic structure (Figure 8) and stereotypy (Figure 9) of song. Between 800 and 1200, fadrozole caused a reduction in acoustic frequency (fundamental and minimum frequency, within-subjects t-tests, $p=0.06$; 1-tailed t-test, $p<0.05$, for both variables) and song stereotypy (bandwidth and entropy stereotypy, within-subjects t-tests, $p<0.05$ and $p=0.06$, respectively; 1-tailed t-test for entropy variance stereotypy, $p<0.05$) compared to vehicle. Fadrozole's effects on minimum frequency persisted from 1200-1400, causing songs to have lower frequency compared to vehicle (within-subjects t-test, $p<0.05$). However, when birds were treated with fadrozole plus estradiol, no perturbations were observed for these measures relative to when they were treated with vehicle (within-subjects t-tests, $p>0.17$ for all comparisons).

Interestingly, when birds were injected with fadrozole they sang songs with higher entropy stereotypy from 1400-1600 compared to when birds were injected with fadrozole plus estradiol (within-subjects t-test, $p<0.05$). This same pattern was observed at 1600-1800 for acoustic frequency (within-subjects t-tests, $p<0.05$ for both fundamental and minimum frequency). There was also a latency effect for energy, wherein fadrozole caused songs to be lower in energy compared to vehicle ($p<0.05$) at 1400-1600; at 1600-1800, fadrozole tended to cause birds to sing songs that were louder compared to when they were treated with fadrozole plus estradiol ($P=0.09$).

Discussion

Many studies on the hormonal regulation of birdsong have centered on assessments of the long-term, genomic effects of sex steroid hormones (Alward et al., 2013; Schlinger and Brenowitz 2002; Walters and Collado, 1991; Sartor et al., 2005; Walters and Harding, 1988). Recently evidence has begun to accumulate indicating that certain behaviors and cognitive processes may be regulated by estrogens acting in a non-genomic fashion (Ervin et al., 2015; Oberlander, et al., 2004; Remage-Healey et al., 2010; Remage-Healey, Jeon, and Joshi, 2013; Remage-Healey and Joshi, 2012; Remage-Healey, 2012; Seredynski et al., 2013). The experiments presented here demonstrate that estrogens may act in this fashion in the regulation of birdsong.

Our first experiment showed that acute aromatase inhibition caused a variety of song features to undergo changes. Fadrozole treatment decreased motivational measures of song, perturbed acoustic features, and decreased song stereotypy on the same day of treatment. In our second experiment we aimed to address how these effects manifested over time on the day of treatment to assess how rapidly these effects occurred and how quickly they dissipated. We showed that for the motivational measures of song, acoustic frequency, and song stereotypy that aromatase inhibition indeed caused rapid perturbations and these effects rebounded rapidly. Interestingly, there was a latency effect for the energy of song, wherein at 1400-1600 fadrozole caused a decrease in energy. This latency effect could be due to relatively long-lasting changes in the activity of the song control circuit. For instance, the global inhibition of aromatase could have

decreased the activation of estrogen receptors in the nucleus intercollicularis (ICo), which projects to areas in the hindbrain that regulate the amplitude of vocalizations. Notably, there is no clear evidence that aromatase-immunoreactive cells are in or nearby ICo, suggesting that the global decrease in estrogen concentration may have led to the decrease in amplitude following fadrozole injection. The fact that this amplitude decrease took place 8 hours after injection could indicate these effects were of a genomic nature.

At the same time, at some of the later time points, fadrozole caused birds to have higher energy, acoustic frequency, and entropy variance stereotypy than when they were treated with fadrozole plus estradiol. As indicated above, songbirds have a remarkable ability to modify their song when auditory feedback is perturbed (Cynx and Rad, 2001; Sakata and Brainard, 2006; Sober and Brainard, 2008). In this case, the perturbed feedback is their produced song that has been altered by acute aromatase inhibition. Thus, the increase in song energy, acoustic frequency, and entropy variance stereotypy could be due to rapid rebound effects due to the bird being able to adaptively modify its song. Alternatively, the increase in song energy and acoustic frequency could be a result of sensorimotor vocal variability due to perturbed song production (Chapter 3; Cynx and Rad 2001) and the rebound in entropy variance stereotypy is the result of the bird modifying song. These hypotheses warrant future investigation.

Collectively, these results suggest that aromatase present in different brain regions is playing an important role in regulating these distinct features of song. For instance, as mentioned above, aromatase is densely expressed in the POM

(Balthazart, et al., 1996) and the conversion of T to estradiol is required for the full activation of singing behavior (Harding, et al., 1988; Walters and Collado, 1991; Walters and Harding, 1988). Based on these observations, it is reasonable to suppose that one possible site at which the acute inhibition of aromatase caused a reduction in the motivation to sing was the POM.

The rapid changes in song in terms of the acoustic features and stereotypy suggest that the aromatase inhibition in the NCM may have led to the perturbations in the acoustic features and song stereotypy. As mentioned above, NCM densely expresses aromatase and those aromatase-expressing neurons project to HVC (Balthazart, et al., 1996; Saldanha et al., 2000). Importantly, Remage-Healey and colleagues (2012) have shown that estradiol produced in NCM transforms the selectivity for a bird's own song in HVC. Hence, the depletion of estrogens caused by fadrozole injection may have caused disruption in this selectivity leading to abnormal song output. Lastly, it is surprising that a feature that was not affected the day of fadrozole injection was affected the day after treatment. One possible explanation, as mentioned above, is that the song disruptions caused early in the day of treatment led the bird to attempt to adapt to these changes. Indeed, while birds can adaptively modify their song over very short time periods (Sakata and Brainard, 2006, Sober and Brainard, 2008) some changes can take place over relatively longer periods; for instance, over 24-48 hours (Sober and Brainard, 2008). Undergoing such adaptive changes could manifest as an increase in entropy.

We hypothesized that the many song features that were affected by fadrozole injections were due to the acute depletion of estrogens at key target brain sites, like the POM and NCM. Indeed when birds were injected with fadrozole plus estradiol this rescued most of the perturbations in song caused by fadrozole injection. Hence, one can conclude that the acute action of estrogens regulate song over very short time periods in a non-gneomic-like fashion. This study provides evidence that birdsong is regulated on a short time scale via the conversion of T to estradiol. These results highlight the multiple ways in which T coordinates multiple facets of birdsong into a functional response.

Figure Legend

Figure 1- Description of the acclimation and injection procedure. VEH-Vehicle; FAD-Fadrozole.

Figure 2- Diagram showing example coronal tissue sections with the A) HPOA present with densely labeled aromatase cells or the B) NCM present with densely labeled aromatase cells. The box present in each figure indicates an example of where microdissections were made. B) The dashed lines indicates the area across which a razor blade was used to remove the hippocampus. Photomicrographs of tissue sections courtesy of Demas et al., (2007).

Figure 3- Effects of fadrozole injection on aromatase activity (AA) over time. Fmol/h-fentomoles per hour. Asterisks indicate a significant difference between the AA in the respective brain regions as a function of treatment. Differences were considered significant at $P \leq 0.05$.

Figures 4 through 6- Effects of acute aromatase inhibition with fadrozole on a variety of song features. Asterisks indicate a significant difference as a function of treatment. Differences were considered significant at $P \leq 0.05$.

Figures 7 through 9- Effects of acute aromatase inhibition with fadrozole and fadrozole plus estradiol on a variety of song features. Asterisks indicate a significant difference as a function of treatment. Differences were considered significant at $P \leq 0.05$.

Figure 1

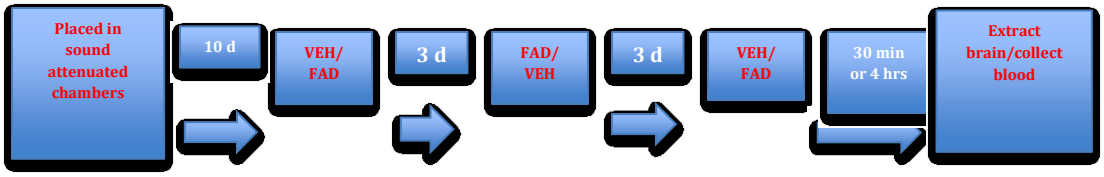
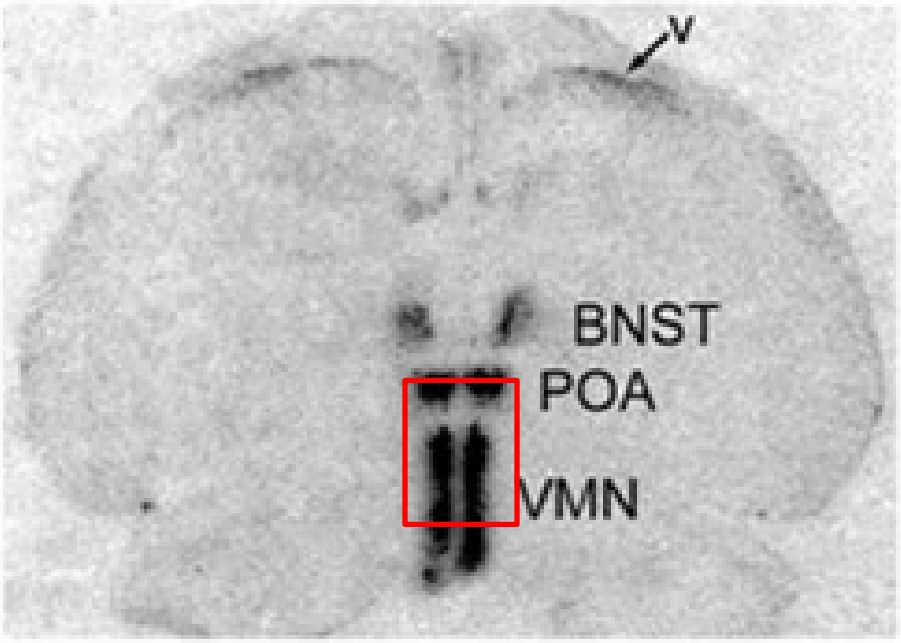


Figure 2

A



B

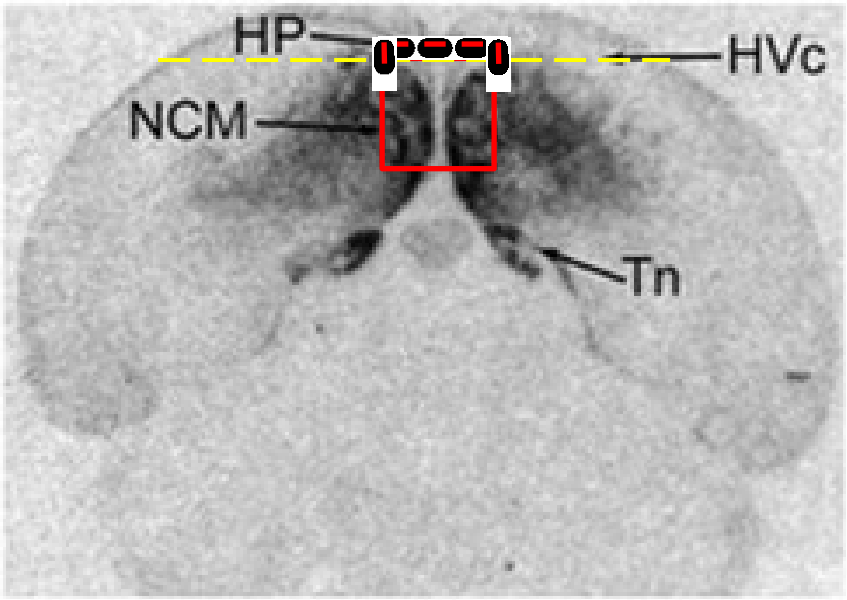


Figure 3

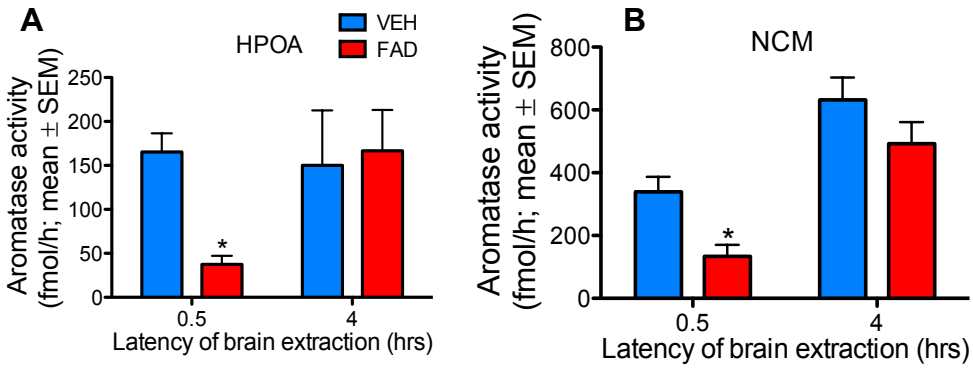

A
HPOA
VEH
FAD
Aromatase activity
(fmol/h; mean ± SEM)
250
200
150
100
50
0
*
0.5
4
Latency of brain extraction (hrs)
B
NCM
800
600
400
200
0
*
0.5
4
Latency of brain extraction (hrs)

Figure 4

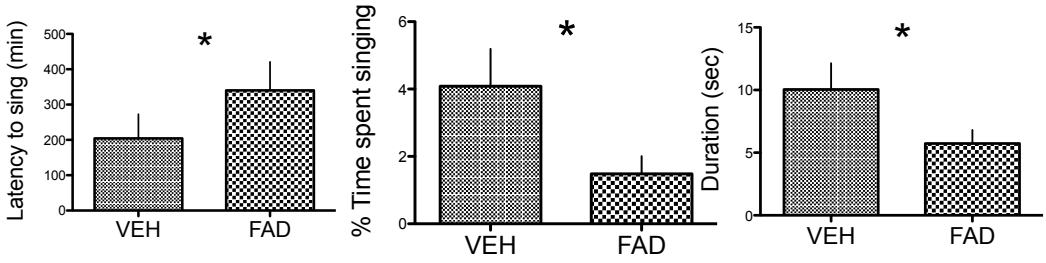


Figure 5

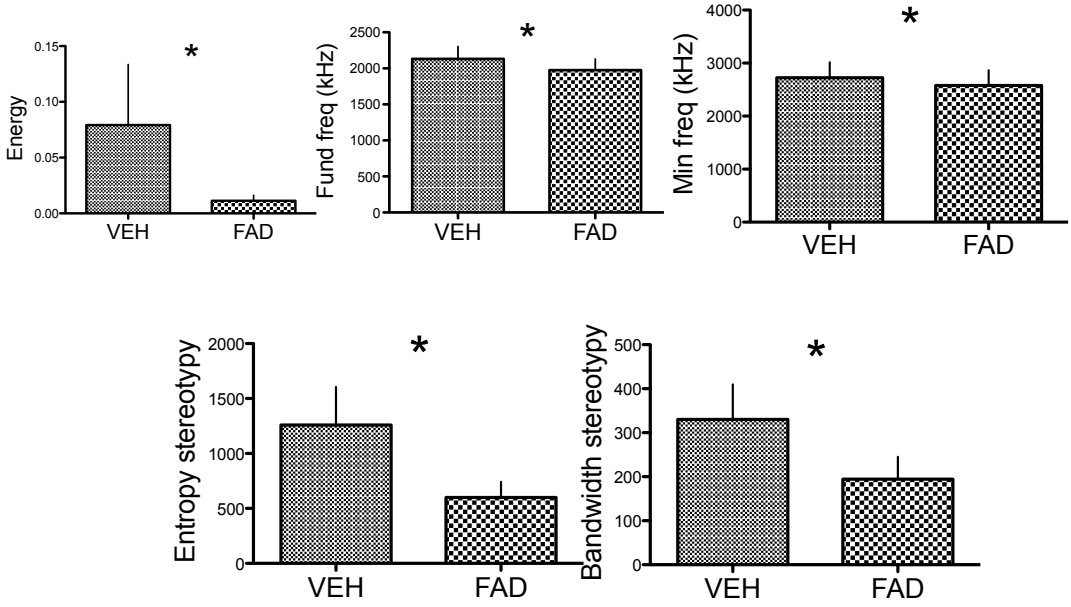


Figure 6

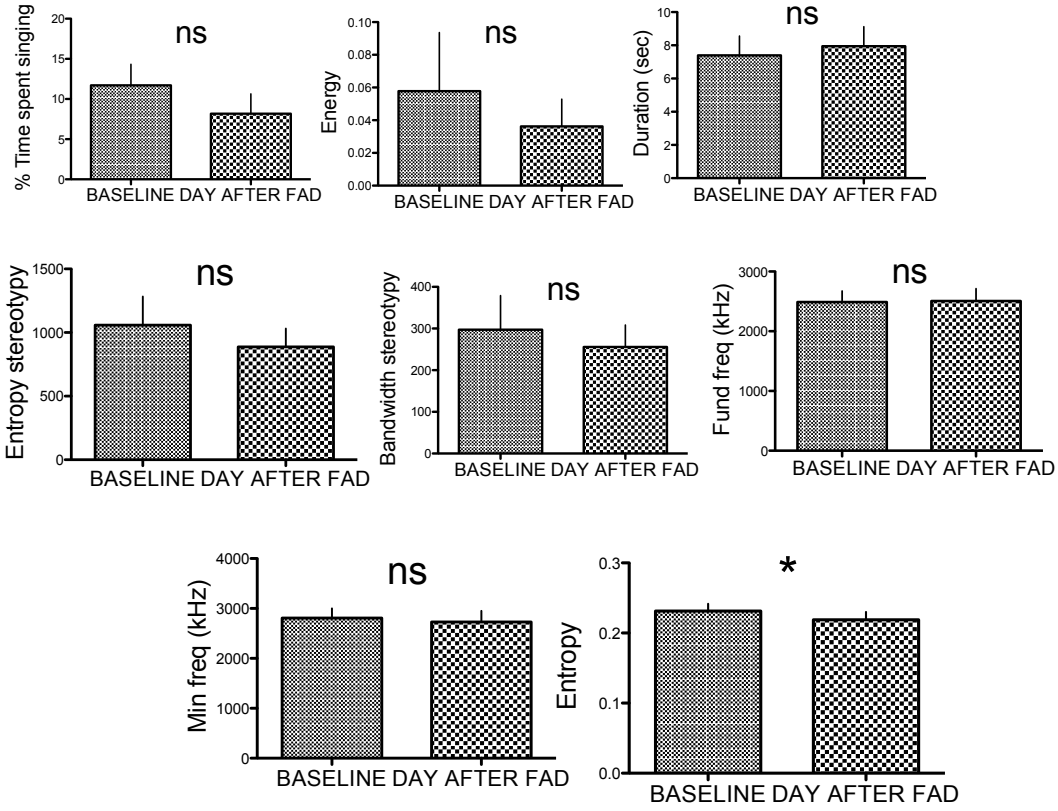

% Time spent singing
ns
BASELINE DAY AFTER FAD
Energy
ns
BASELINE DAY AFTER FAD
Duration (sec)
ns
BASELINE DAY AFTER FAD
Entropy stereotypy
ns
BASELINE DAY AFTER FAD
Bandwidth stereotypy
ns
BASELINE DAY AFTER FAD
Fund freq (kHz)
ns
BASELINE DAY AFTER FAD
Min freq (kHz)
ns
BASELINE DAY AFTER FAD
Entropy
*
BASELINE DAY AFTER FAD

Figure 7

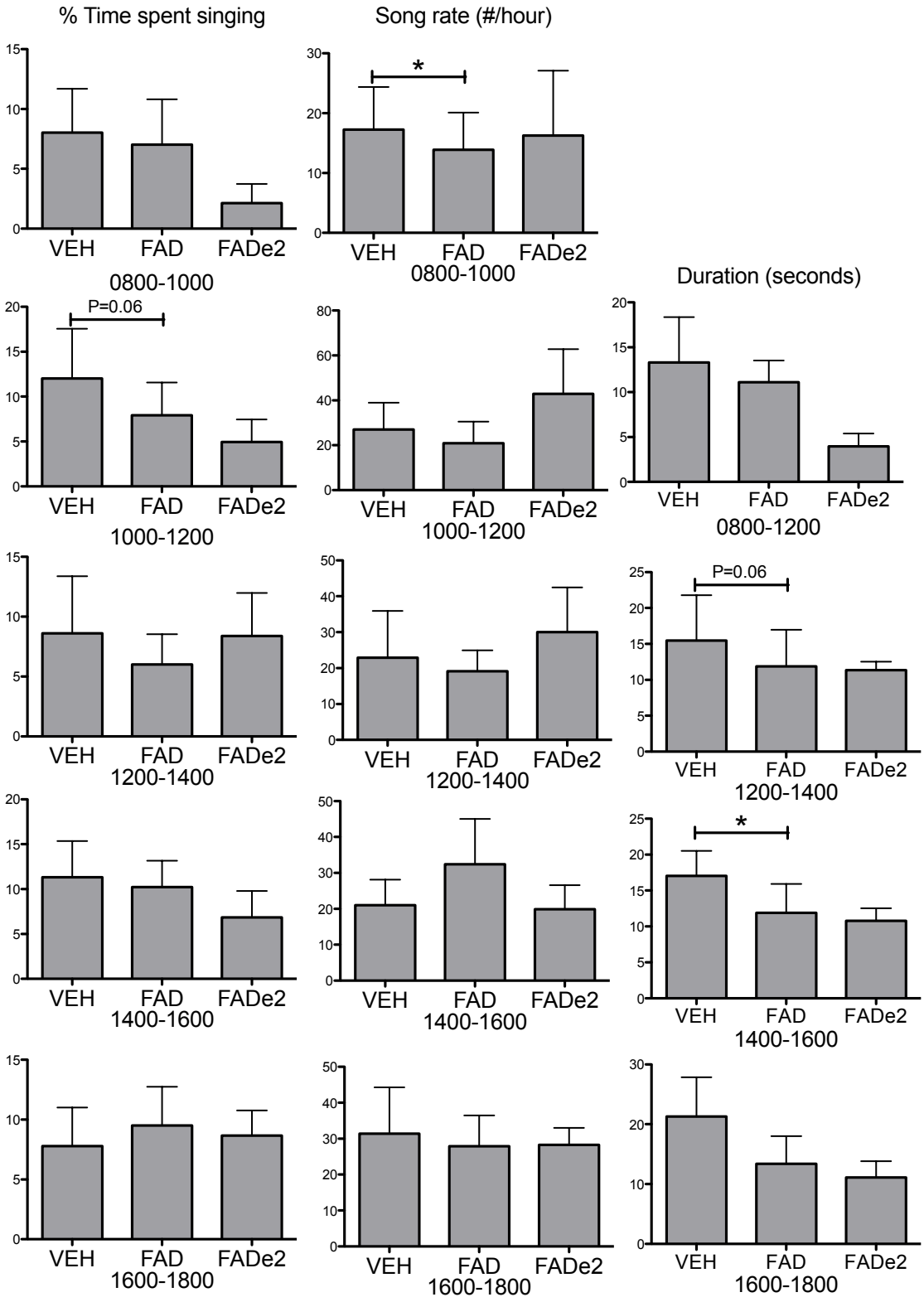

% Time spent singing
Song rate (#/hour)
Duration (seconds)
VEH
FAD
FADe2
0800-1000
1000-1200
1200-1400
1400-1600
1600-1800
0800-1200
P=0.06
*

Figure 8

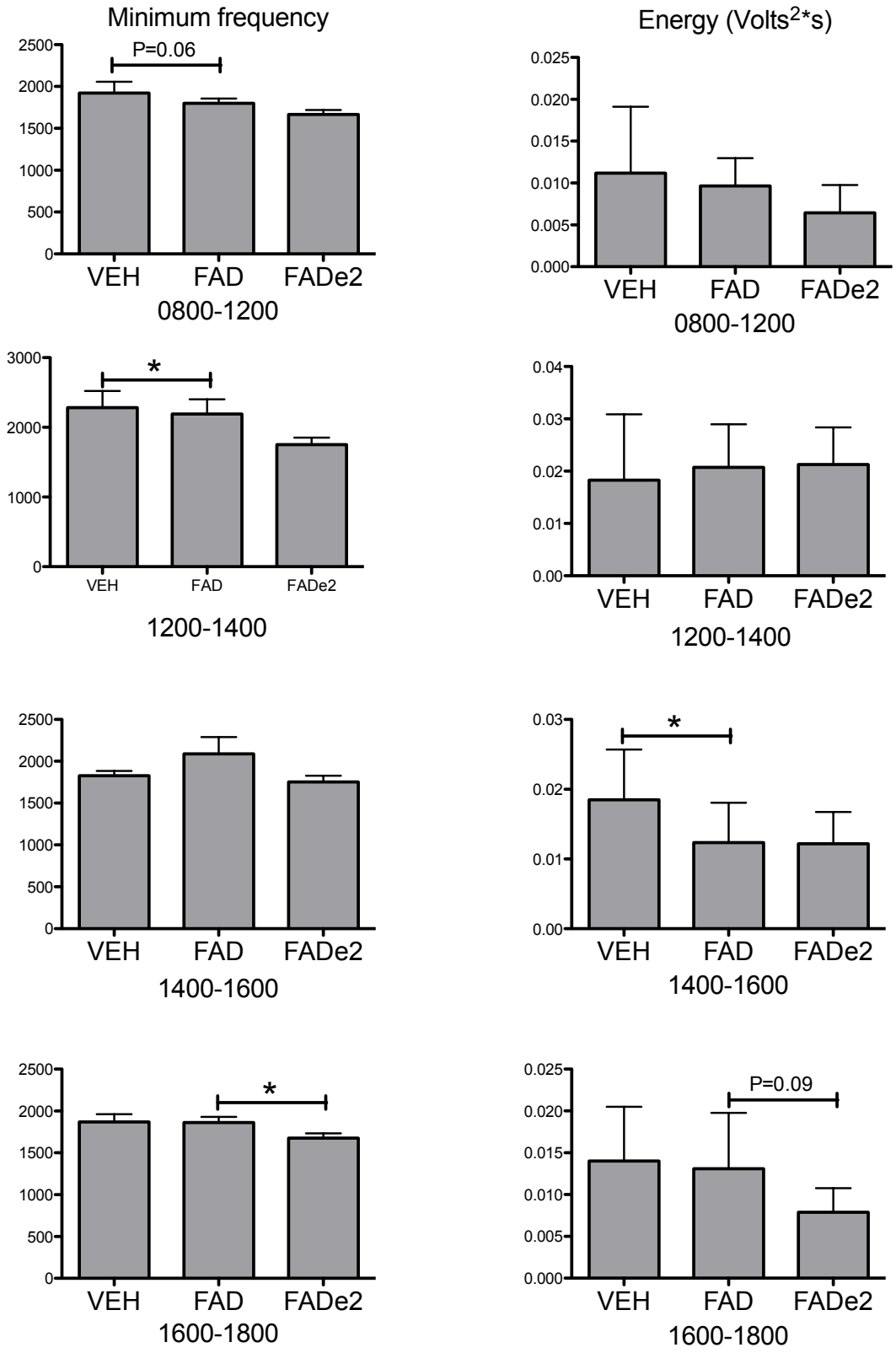
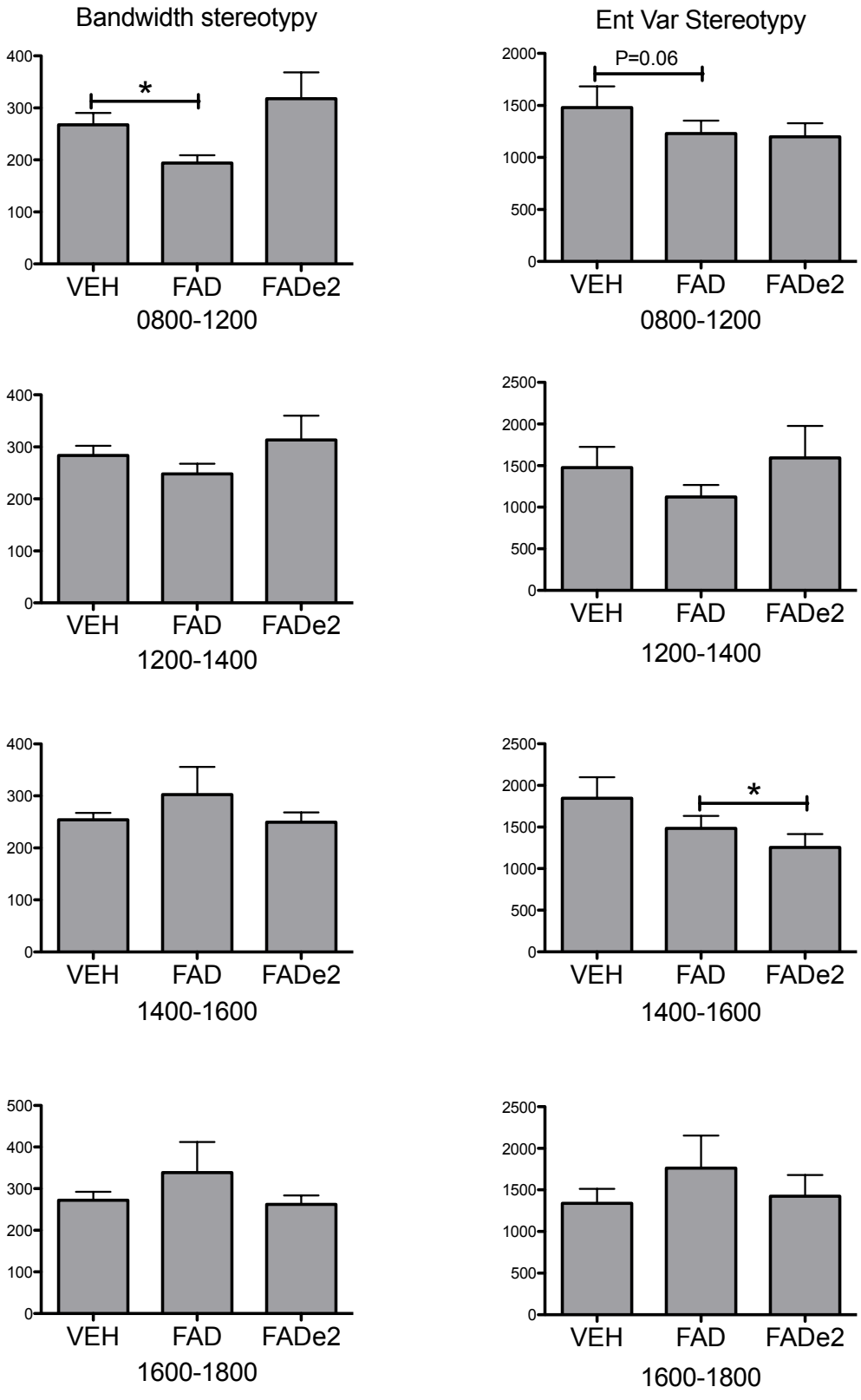

Minimum frequency
Energy (Volts2*s)
P=0.06
VEH
FAD
FADe2
0800-1200
*
1200-1400
1400-1600
P=0.09
1600-1800

Figure 9


Bandwidth stereotypy
Ent Var Stereotypy
P=0.06
*
VEH
FAD
FADe2
0800-1200
1200-1400
1400-1600
1600-1800

Chapter 9: Final summary and concluding remarks

For decades oscine songbirds (i.e. members of the suborder passeres) have been used as one of the primary non-human animal model for studying how complex vocalizations are learned and the underlying neural basis that generates these complex vocalizations (Brainard and Doupe, 2013; Brenowitz et al., 2010; Fee and Scharff, 2010; Marler, 1970; Nottebohm and Liu, 2010; Riede and Goller, 2010). Species in this suborder possess a network of discrete nuclei with distinct functions to control this behavior and consequently songbirds are among the most well-studied non-human animals. One of the most striking attributes of songbirds from the temperate zone is the robust seasonal change in song that occurs as well as changes in the underlying neural substrate that regulates song, both of which have a clear link to the corresponding changes in hormonal milieu. However, only somewhat vague assumptions were made about the precise role played by hormones in activation of song behavior, and little attention was given to the question of where these hormones act to modify song behavior. Some researchers made claims such as T 'activates the song control system' to stimulate singing and only a few researchers suggested T is working in a more non-redundant manner to regulate the different attributes of song and few acknowledged that T may actually act outside of the traditional SCS to regulate the motivational aspects of song. The results of the studies in this thesis collectively demonstrate that the role of hormonal action in the songbird brain on song and neuroplasticity is much more complex than originally thought. The results of the site specificity of T action in the regulation of song are presented below (Summary Table).

In the first study I investigated how a photoperiodic, T-dependent behavior, birdsong, and the associated new neuron incorporation into the song nucleus HVC, may be tempered by the social context. Indeed, while long days induced similar reproductive states in all birds, the presence of a female drastically reduced song output and the acoustic measures associated with sexually-motivated song but at the same time this group incorporated more neurons into HVC than birds that were singing robustly. These results suggest singing and social context have dissociable effects on neuroplasticity and these effects may work through different mechanisms.

The next several studies used hormonal implantation and receptor blockade techniques to address the anatomical specificity of T action in the regulation of song attributes and neuroplasticity. Indeed, in chapter 3 it was shown that the activation of androgen receptors in the periphery, presumably via the syrinx, is necessary to sustain the complexity of song and intricately involved in the quality production of strongly sexually-selected vocal signals within songs, trills. However, androgen receptor activation at the syrinx is not involved in regulating the motivation to sing or song stereotypy. These results have important implications for the evolution of vocal signals used in socio-sexual interactions and sexual selection in general (Podos, 1997).

However, in chapter 4 it was demonstrated that T in the POM is a critical site for the activation of the motivational measures of song but T must act elsewhere, such as areas in the SCS like HVC, RA, and the syrinx to regulate measures of song quality such as acoustical consistency (stereotypy) and complexity as well as song energy. Moreover, this study provided support for the hypothesis that singing activity can drive plasticity in the SCS.

This led to the experiments conducted in chapters 5 and 6. For instance, in chapter 5 it was shown that T in HVC is critical for the regulation of song stereotypy and partially involved in the regulation of song complexity. We then investigated further whether trills within the songs of canaries were differentially regulated by T in the HVC versus POM in chapter 6. These results indicated that T in the HVC is only partially or not involved in regulating most of the features of these sexually-relevant vocal signals. Overall, these results indicated that T elsewhere, such as in the syrinx, as shown in chapter 3, and/or in RA may be involved in regulating the complexity of song and complexity and stereotypy of trills. Furthermore, in chapter 5 we investigated how T and singing differentially affect the neuroplasticity in the SCS, as measured by gross volumetric changes and the incorporation of new neurons in HVC. Here we showed that T can directly (T in HVC) as well as indirectly (via activation of singing activity) and possibly interactively modulate features of neuroplasticity. Indeed, we showed that T in HVC may lead to a recruitment of new neurons into HVC as well as the incorporation of new neurons into HVC; another factor, most likely singing activity also is involved in increasing the incorporation of new neurons into HVC.

In chapter 7, the role of androgen receptor activation in HVC versus activation in RA was investigated. These results showed that androgen receptors in HVC play a role in the regulation of song and trill stereotypy, replicating the results from chapters 5 and 6; it was also shown that androgen receptors in RA are critical in the regulation of song stereotypy, but are also involved in the regulation of complexity. Combined with the results from chapter 5, it appears that androgens acting in HVC can transsynaptically activate RA to regulate features of song stereotypy, but androgen receptor activation in

RA is permissive for this transsynaptic regulation to occur (Meitzen et al., 2007). Given the more subtle effects of androgen receptor blockade in HVC suggests that both metabolites of T, such as DHT and estrogens, are both involved in regulating song and trill stereotypy (Meitzen et al., 2007).

Lastly, the results of the fadrozole and fadrozole+estradiol injection studies in canaries indicate song is regulated over relatively short time periods by estrogens, possibly by non-genomic mechanisms. For instance, acute aromatase inhibition caused perturbations in the motivational, acoustic, and stereotypy measures of song on the same day of treatment with fadrozole but these differences were gone by the next day. In the subsequent experiment, estradiol was able to rescue the song perturbations caused by fadrozole injection and estradiol depletion caused by fadrozole caused rapid, short-lasting perturbations in song. Some of the features affected were also affected in castrated canaries that only received T in their POM, suggesting that the reduction in aromatase activity in regions like POM and NCM caused by castration played a role in the perturbed song in POM-T birds.

Conclusion

Birdsong is a complex, learned vocal behavior regulated by a system of discrete brain regions that is modulated by a variety of other brain regions. The experiments described in this dissertation show the intricate manner by which steroid hormones like testosterone regulate this complex behavior. Overall, the regulation of birdsong and the neural substrates that subserve this behavior by steroid hormones is highly non-redundant, with androgens and estrogens most likely acting at discrete levels of the

song control system to orchestrate the different features of song into a functional, adaptive response. Outstanding questions still remain, however.

For instance, we did not find a clear indication of where steroid hormones are acting to regulate the amplitude of songs. One candidate set of AR-expressing brain regions including respiratory hindbrain nuclei, such as Ram and the ventral respiratory group (VRG). Intriguingly, Ram densely expresses androgen receptors and is involved in regulating the coordination of respiratory activity with song production and song amplitude. Another possible site involved is the ICo, which converges onto Ram and Pam.

Interestingly, multiple experiments presented above showed that AR blockade or aromatase inhibition led to an increase in the amplitude of vocalizations. We have hypothesized that these amplitude increases, combined with the observation of increases in vocalization pitch and duration, are the result of adaptive song modification following song perturbation. This interpretation is in in line with what may characterize a state of what occurs when animal experiences perturbed feedback, where increases in amplitude, pitch, and/or duration are the result of vocal exploration involved in the adaptive song modification process. In light of the results in chapters 5 and 6, these results indicate that steroid hormones acting at a site other than HVC or RA may regulate this process. A possible site could be LMAN, a brain region critical for sensorimotor vocal exploration involved in song learning, crystallization, and maintenance. Importantly, recent results from our lab (Rouse and Ball, 2015) showed that female canaries treated with T but with lesioned LMAN do not undergo the normal song learning process and also show less variation in song energy compared to birds

with an intact LMAN. Indeed, experiments investigating the role of androgens in LMAN in the regulation of song are especially justified.

Overall though these studies demonstrate how steroid hormones can be valuable tools in dissecting the function of different targets in a neural circuit. It also illustrates how one must view steroids as pleiotropic in nature. Indeed, while it has been generally accepted that steroids orchestrate a variety of traits in morphology, physiology, and behavior, this thesis clearly shows that steroid hormones can also act throughout different parts of the brain to integrate different features of a single behavior, birdsong, into a functional response. This type of pleiotropic regulation by steroids has been discussed by McCarthy and Arnold (2011), but in the context of the organizing effects of steroids during early development. It will be interesting to investigate the molecular underpinnings of the regulation of the distinct parts of song and neuroplasticity. Some candidate molecular substrates exist as underlying these changes, such as BDNF (Hartog et al., 2009) as well as the glutamatergic system (White et al., 1999). Moreover, it is likely the fast-acting regulation of songs by estrogens are via membrane-bound ER (Seredynski et al., 2014) possibly ER coupled to metabotropic glutamide receptors (Meitzen and Mermelstein, 2011). These possibilities should be investigated in future experiments.

Finally, it is interesting that the regulation of birdsong and its underlying neuroplasticity by T is distributed in such a non-redundant manner throughout the brain. One possible answer is that this allows for more computational power over the regulation of birdsong. For instance, birdsong is highly seasonal in its production and function, and the importance of song acoustic structure, variation, learning, and overall

output varies based on season, context, and the current state of the animal. Thus, it would be critical to distribute the different features of song sensitive to steroid hormones throughout different parts of the SCS. A clear example of the state of one song control region modulating the input of another is the fact that RA interneurons with a GABA phenotype modulate the input from HVC and LMAN, providing further gating of the signals from these nuclei before the signal for singing eventually converges onto the syrinx (Spiro et al., 1999). Thus, the non-redundant actions of T in the regulation of birdsong highlight a more general principle about the regulation of complex behaviors by steroid hormones.

Summary Table: Regional specificity of T in the regulation of birdsong

Feature	PER-T	HVC-POM T	HVC AR	RA AR	POM-T	Syrinx AR
Song Rate	+	-	-	-	+	-
Song Stereotypy	+	+	+/-	+	-	-
Song Complexity	+	+/-	-	+	-	+
Trill production	+	+	-	-	+	-
Trill repetition rate	+	+/-	-	-	-	+
Trill stereotypy	+	+/-	+	+	-	+*
Trill complexity	+	+/-	-	+	-	+
Loudness	+	-	-	-	-	-

This summary table shows how T and androgen receptors (AR) in various regions of the SCS regulate song. A '+' indicates that T or AR in the specific region is involved in regulating the features in the first column. A '-' indicates non-involvement. A '+/-" indicates there is evidence that T or AR in the specific region appears to be partially involved in regulating a particular feature. The +* in the Syrinx AR column for Trill stereotypy denotes that syrinx AR may be involved in keeping variability of trills important for mate attraction at high levels.

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Appendix

Song acoustic features of interest*
Energy (measure of amplitude)
Fundamental frequency (measure of pitch)
Minimum frequency
Maximum frequency
Bandwidth (max freq minus min freq)
Entropy (signal noise)
Fundamental frequency variance**
Minimum frequency variance
Maximum frequency variance
Bandwidth variance
Entropy variance

*Stereotypy, a measure of the consistency of a feature across song renditions, was computed for measures of acoustic frequency and variance.

**Variance measures are gross indicators of changes in the acoustic content of songs and can be used as a measure of song complexity.

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RESEARCH INTERESTS

I am interested in how the brain integrates across distinct neural circuits to give rise to complex behaviors and how neuromodulatory factors such as hormones regulate these circuits.

EDUCATION

2011 – 2015 Ph.D.		**Johns Hopkins University** Department of Psychological & Brain Sciences
2011 – 2013 M.A.		**Johns Hopkins University** Department of Psychological & Brain Sciences
2008 - 2010 B.S.	**(Honors)**	**University of California, Davis** Neurobiology, Physiology, and Behavior

RESEARCH & PROFESSIONAL EXPERIENCE

6/11 – present Graduate Research Assistant, **Behavioral Neuroendocrinology Lab** (PI: Gregory Ball)
Johns Hopkins School of Arts and Sciences
Research topics: Understanding the neural and hormonal regulation of birdsong and accompanying neuroplasticity in songbirds. I use global and targeted hormonal manipulations combined with acoustic analysis, histological and molecular techniques to address these questions.

9/08 – 7/11 Independent Research, **Animal Communications Lab**, (PI: Thomas Hahn)
University of California, Davis
Research topics: Investigating the organization and patterns of learned vocalizations in a complex vocal learner, the pine siskin. I also quantified the presence of heterospecific vocal

mimicry within the birds' songs. I also performed general song analysis for this project.

6/10 – 5/11 Behavioral Therapist, **Center for Autism and Related Disorders**

Sacramento, C.A.
Responsibilities: Provided services to children with autism spectrum disorders. Used applied behavioral analysis to increase the quality of speech and frequency of adaptive behaviors, while decrease stereotypic/ritualistic and maladaptive behaviors.

3/9 – 9/10 Assistant Researcher, **Genetics Medicine**, (PI: Joseph Devaney)
Children's National Medical Center, Washington, D.C.
Research topics: Investigated the molecular underpinnings of diabetes and obesity. Specifically, used genotyping to determine alleles linked to diabetes and obesity. Performed and trained colleagues on DNA isolation and quantification and genotyping to address these topics.

LABORATORY TECHNIQUES

Small animal surgeries, brain cannulations, central and peripheral pharamcological manipulations, immunohistochemistry, neural tract tracing, polymerase chain reaction, genotyping, western and southern blot, Nissl staining, brain morphometric analysis, cellular counting techniques, acoustic analysis, behavioral analysis.

TEACHING

9/14 – 1**2/14** Teaching Assistant, Behavioral Endocrinology (upper level course, ~70 students), Drs Gregory Ball and Farrah Madison.

9/13 – 12/13 Teaching Assistant, Research Methods in Experimental Psychology (upper level course, ~80 students), Dr. Howard Egeth.

2/13 – 5/13 Teaching Assistant, Animal Behavior, (upper level course, ~90 students) Drs Gregory Ball and Farrah Madison.

9/12 – 12/12 Teaching Assistant, Behavioral Endocrinology, (upper level course, ~70 students) Drs Gregory Ball and Farrah Madison.

2/12 – 5/12 Teaching Assistant, Psychopharmacology, (upper level course, ~100 students) Dr. Linda Gorman

HONORS & AWARDS

2010 Citation for Outstanding Performance in Neurobiology, Physiology, and Behavior
2009 – present Phi Kappa Phi Honor Society
2009 – present Phi Sigma Biological Sciences Honor Society
2008 – 2009 Dean's List, UC Davis

ACADEMIC SERVICE

2013 – 2014 Colloquium Committee for Psychological & Brain Sciences Department

2008 – 2009 Neurobiology, Physiology, and Behavior Club
Volunteered for the local community to educate them on brain and behavior. To this end, I prepared diagrams and posters during events like Brain Awareness Week and Davis, CA Picnic Day to educate them and brain disorders and brain and behavior dimorphisms between males and females.

PUBLICATIONS

Alward, B.A., Mayes, W. D., Peng, K., Stevenson, T. J., Balthazart, J., & Ball, G. F. (2014). Dissociable effects of social context on song and doublecortin immunoreactivity in male canaries. *European Journal of Neuroscience*, *40*(6), 2941-2947.

Alward, B. A., Balthazart, J., & Ball, G. F. (2013). Differential effects of global versus local testosterone on singing behavior and its underlying neural substrate. *Proceedings of the National Academy of Sciences*, *110*(48), 19573-19578.

CONFERENCE PRESENTATIONS

Alward, B.A., Madison, F.N., Gravley, W.T., Ball, G.F. (2015) The effects of bicalutamide, a peripherally-selective androgen receptor blocker, on canary song. Poster presented at the annual meeting for the Society of Behavioral Neuroendocrinology.

Alward, B.A., Balthazart, J., and Ball G.F. (2014) Anatomical specificity in the action of testosterone in the regulation of song and underlying neuroplasticity in canaries. Poster presented at the annual Birdsong Satellite meeting at the Society for Neuroscience.

Alward, B.A., Chan, T.T. Balthazart, J , Cornil, C., and Ball, G.F. (2014) Evidence for fast, non-genomic-like actions of estrogens in the regulation of birdsong. Poster presented at the annual meeting to the Society for Neuroscience.

Madison, F.N. Alward, B.A. Ball, G.F. (2014) Investigating possible intraspecific variation in testosterone-induced neuroplasticity by comparing two canary breeds. Poster presented at the annual meeting to the Society for Neuroscience.

Yoder, K.M., Iyilikci, O., Alward, B.A., Ball, G.F. (2014) Distribution of serotonergic markers in the brains of Japanese quail, European starlings, and zebra finches. Poster presented at the annual meeting to the Society for Neuroscience.

Alward, B.A., Balthazart, J., and Ball, G.F. (2014) Anatomical specificity in the action of testosterone in the regulation of song and underlying neuroplasticity in canaries**.** Accepted presentation at the International Conference on Hormones, Brain, and Behavior held at the University of Liege in Liege, Belgium.

Alward, B. A., Balthazart, J., & Ball, G. F. (2013). Differential effects of global versus local testosterone on singing behavior and its underlying neural substrate. Poster presented at the annual meeting to the Society for Neuroscience.

Alward B.A., Balthazart J., Ball G.F. (2013) Testosterone, song, and sex in canaries: the role of the medial preoptic nucleus. Poster presented at the annual meeting for the Society of Behavioral Neuroendocrinology.

Iyilikci O., Alward B.A., Balthazart J., Ball G.F. (2013) Localization of the nucleus accumbens in Japanese quail and European starlings based on hodological, immunohistochemical and functional criteria. Poster presented at the annual meeting for the Society of Behavioral Neuroendocrinology.

Alward B.A., Balthazart J., Ball G.F. (2013) Testosterone, song, and sex in canaries: the role of the medial preoptic nucleus. Accepted presentation at the North American Society for the Study of Comparative Endocrinology held at the Universidad Nacional Autonoma de Mexico in Queretaro, Mexico.

Alward B.A., Rouse M.L., Stevenson T.J., Ball G.F. (2012) Photoperiodic and social modulation of song output and structure in Border canaries (*Serinus canaria*). Presentation given at the Society for Integrative and Comparative Biology Conference, Charleston, S.C.

Alward, B. A., Stevenson, T. J., Peng, K., Rouse M.L., Mayes, W. D., Balthazart, J., & Ball, G. F. (2012). Effects of social context and song on doublecortin expression in the HVC of canaries. Poster presented at the annual meeting to the Society for Neuroscience.

Alward B.A., Stevenson T.J., Ball G.F. (2012) Distribution of the photoreceptor neuropsin in the canary brain. Poster presented at the annual meeting for the Society of Behavioral Neuroendocrinology.

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